

Anti-proliferative activity and chemoprotective effects towards DNA oxidative damage of fresh and cooked Brassicaceae

Lisa Ferrarini¹, Nicoletta Pellegrini², Teresa Mazzeo², Cristiana Miglio^{2,3}, Serena Galati¹, Francesco Milano¹, Carlo Rossi¹ and Annamaria Buschini^{1*}

¹Department of Genetics, Biology of Microorganisms, Anthropology, Evolution, University of Parma, Parco Area delle Scienze 11/A, Parma 43100, Italy

²Department of Public Health, University of Parma, Parma 43100, Italy

³Food and Nutrition National Research Institute (INRAN), Rome 00178, Italy

(Submitted 3 February 2011 – Final revision received 9 June 2011 – Accepted 7 July 2011 – First published online 17 November 2011)

Abstract

Epidemiological evidence shows that regular consumption of Brassicaceae is associated with a reduced risk of cancer and heart disease. Cruciferous species are usually processed before eating and the real impact of cooking practices on their bioactive properties is not fully understood. We have evaluated the effect of common cooking practices (boiling, microwaving, and steaming) on the biological activities of broccoli, cauliflower and Brussels sprouts. Anti-proliferative and chemoprotective effects towards DNA oxidative damage of fresh and cooked vegetable extracts were evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and Comet assays on HT-29 human colon carcinoma cells. The fresh vegetable extracts showed the highest anti-proliferative and antioxidant activities on HT-29 cells (broccoli > cauliflower = Brussels sprouts). No genotoxic activity was detected in any of the samples tested. The cooking methods that were applied influenced the anti-proliferative activity of *Brassica* extracts but did not alter considerably the antioxidant activity presented by the raw vegetables. Raw, microwaved, boiled (except broccoli) and steamed vegetable extracts, at different concentrations, presented a protective antioxidative action comparable with vitamin C (1 mM). These data provide new insight into the influence of domestic treatment on the quality of food, which could support the recent epidemiological studies suggesting that consumption of cruciferous vegetables, mainly cooked, may be related to a reduced risk of developing cancer.

Key words: *Brassica* vegetables: Cooking methods: Chemoprevention: Comet assay

Epidemiological studies have shown that regular consumption of *Brassica* vegetables is associated with a reduced risk of some chronic diseases; in particular, gastric or lung cancers and CVD^(1,2). *Brassica* vegetables contain a wide range of natural chemopreventive agents, such as folate, fibre, vitamin C, tocopherols, polyphenols, carotenoids and chlorophylls^(3–6). Furthermore, cruciferous vegetables are a rich source of glucosinolates, sulphur-containing compounds, whose hydrolysis results in the formation of biologically active compounds, including indoles and isothiocyanates^(7–9). All these molecules can retard the development and progression of precancerous cells into malignant cells⁽¹⁰⁾ acting through different mechanisms and in different compartments, but they are thought to be mainly free radical scavengers⁽¹¹⁾.

It is known that cooking induces significant changes in chemical composition, influencing the concentration and availability of bioactive compounds in vegetables.

Both positive and negative effects have been reported depending upon the differences in process conditions and morphological and nutritional characteristics of the vegetable species^(6,12–16). The cooking of vegetables can decrease water-soluble and heat-sensitive nutrients, such as vitamin C, but also can lead to disruption of the food matrix and dissociation of some compounds from the plant matrix components. This determines the release of plant components, which, in turn, can improve their digestion and absorption, increasing their bioavailability^(17–19).

Although some of the vegetables consumed in the human diet are processed to be eaten, there are few works that correlate their biological activity on human cell lines *in vitro* to cooking treatments. Recently, the effect of grilling and boiling processes on eggplant showed a positive effect on antioxidant concentrations and chemopreventive activities of the human polymorphonuclear neutrophils⁽²⁰⁾.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; g eq ww/ml, equivalent of wet weight g/ml; LED, lowest effective dose; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; TI, tail intensity.

* **Corresponding author:** Professor A. Buschini, fax +39 0521 905604, email annamaria.buschini@unipr.it

There is an increasing attention in the evaluation of the antiproliferative effects induced by vegetable extracts on human cancer cell lines, performed mainly by cytotoxicity tests such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays^(21,22). Furthermore, an interesting method for studying the effects of foods on the risk of cancer is the Comet assay, a single-cell gel electrophoresis technique for measuring DNA breakage in individual cells⁽²³⁾. The comet assay is performed mainly to evaluate the effects of antioxidants *in vitro*^(24,25) or in intervention studies⁽²⁶⁾.

The goal of the present study was to evaluate the effect of common cooking practices (i.e. boiling, microwaving and steaming) on the biological activities of broccoli, cauliflower and Brussels sprouts. The anti-proliferative activity and chemoprotective effect towards DNA oxidative damage of fresh and cooked vegetable extracts were evaluated by MTS and Comet assays.

Experimental methods

Chemicals

All chemicals were of analytical grade or they complied with the standards required for tissue culture experiments. Reagents for electrophoresis, normal melting point (1%) and low melting point (0.7%) agarose, dimethylsulfoxide, ethidium bromide, ascorbic acid and general laboratory chemicals were from Sigma-Aldrich Company Limited (Milan, Italy). The cell culture medium and reagents were from BioWhittaker (Lonza, Milan, Italy). Stabilised H₂O₂ (3%) was from Zeta Farmaceutici (Vicenza, Italy).

Vitamin C: stock solutions of 100 mM ascorbic acid were prepared fresh in a modified Krebs buffer, containing 137 mM-NaCl, 5.4 mM-KCl, 2.8 mM-CaCl₂, 1 mM-MgSO₄, 0.3 mM-NaH₂PO₄, 0.3 mM-KH₂PO₄, 10 mM-glucose, 10 mM-HEPES and 10 mM-Tris, pH 7.4. Dilutions of these stock solutions were made directly in the cell culture media.

Preparation of vegetables

Freshly harvested broccoli (*Brassica oleracea* L. cv. *Italica*), Brussels sprouts (*B. oleracea* L. cv. *gemmifera*) and white cauliflower (*B. oleracea* L. cv. *botrytis cauliflora*) of a single batch were analysed. Fresh Brussels sprouts were purchased from a local market on the day of processing after road transport under refrigerated conditions within 24 h of harvest and stored at 7°C for up to 1 d before they were taken. Fresh broccoli and cauliflower were obtained by a local purchaser and analysed within 3 d of harvest. The samples were stored at 4°C before analysis.

Fresh broccoli and cauliflower were cleaned by removing of inedible parts and then chopped into homogeneous pieces, leaving a stem of 2.5 cm. Brussels sprouts were deprived of the external leaves.

To obtain more homogeneous samples, each vegetable was prepared in batches of 500 g. Each batch was then divided into

five equal portions. One portion was retained raw; the others were cooked in four different methods in triplicate, as given below.

Cooking treatments

Cooking conditions were optimised by preliminary experiments carried out for each vegetable. For all cooking treatments, the minimum cooking time to reach tenderness for adequate palatability and taste, according to Italian eating habits, were used.

Boiling

Brassica vegetables were added to boiling tap water in a covered stainless steel pot (1:5, food/water) and cooked on a moderate flame. Cooking time was 8, 10 and 10 min for broccoli, Brussels sprouts and cauliflower, respectively. For each cooking trial, ten samples were boiled. Then, the samples were drained off for 30 s.

Steaming

Two types of steaming equipment were employed: an air/steam impingement oven and a domestic cooker equipped with a mesh basket. Air/steam oven treatments were carried out in a Combi-Steal SL oven (V-Zug, Zurich, Switzerland). Nine specimens of vegetables were placed in the oven equilibrated to room temperature before each cooking trial. Eight samples were arranged in a circle, and one put at the centre to ensure uniform heating conditions in all samples for each cooking trial. Cooking time was 13, 17 and 13 min for broccoli, Brussels sprouts and cauliflower, respectively. The samples were put into the oven when a temperature of 100°C was reached (displayed by the apparatus).

A single layer of nine specimens of *Brassica* vegetables was steamed in a domestic closed vessel using a stainless steel steam basket suspended above a small amount of boiling water. Cooking time was 15, 18 and 11 min for broccoli, Brussels sprouts and cauliflower, respectively.

Microwaving

Microwave treatments were carried out in a domestic microwave oven (Samsung Electronics Company Limited, Paldal-Gu Suwon Kyungki-Do, Korea) without water. Ten specimens of vegetables were exposed at a frequency of 2450 Hz at low power (300 W) on the rotatory turntable plate of the oven. Cooking time was 30, 18 and 30 min for broccoli, Brussels sprouts and cauliflower, respectively.

After all cooking experiments, the samples were cooled rapidly on ice and then freeze-dried utilising a Brizzio-Basi instrument (Milan, Italy). The dried sample material was finely ground, kept in sealed bags, and stored at -20°C until the analysis.

Vegetable extract preparation

Chemical analyses of the phytochemical compound content in fresh and cooked samples were performed before the

preparation of the extracts. For a complete description see Pellegrini *et al.*⁽²⁷⁾. A synthesis of the results is reported in Fig. 1.

Aqueous extracts were prepared by mixing the dried samples with Dulbecco's modified Eagle's medium (DMEM; supplemented with 1% glutamine, 0.5% penicillin/streptomycin and 10% fetal bovine serum). The suspensions were further subjected to Stomaker treatment for 6 min, a centrifugation step (20 min, 4°C and 15 000 *g*) and the supernatants were sterile-filtered (0.20 µm syringe filter; SARSTEDT,

Nümbrecht, Germany) and divided into aliquots (stored at –20°C). The relative concentration of each sample, equivalent of wet weight g/ml (geq ww/ml), was calculated by dividing the wet weight for the volume (ml) of the extract obtained after the centrifugation and filtration steps^(28,29).

Cell lines

The colon is the major cancer site thought to be protected by vegetables, so for *in vitro* studies we employed a cell line, HT-29, used widely as a model for colon cancer.

The human colon adenocarcinoma cell line HT-29 was kindly obtained from the Northern Ireland Centre for Food and Health. Prior to the experiments, the cells were thawed and grown in tissue culture flasks as a monolayer in DMEM, supplemented with 1% glutamine, 0.5% penicillin/streptomycin and 10% fetal bovine serum at 37°C in a humidified (95% CO₂ (5%) incubator. The cultured cells were trypsinised with trypsin/EDTA for a maximum of 5 min and seeded with a subcultivation ratio of 1:3–1:8. The cell numbers and viabilities were determined with the trypan blue exclusion test.

Antiproliferative activity

Cell viability was detected by CellTiter96^R AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). This test contains a tetrazolium compound (MTS, inner salt) and an electron coupling reagent (phenazine ethosulfate). The MTS tetrazolium compound is bioreduced by cells into a coloured formazan product that is soluble in a tissue culture medium. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells.

In order to determine cell viability, in the exponential phase of growth, the cells were seeded at 5×10^4 /ml in ninety-six-well plates, in DMEM supplemented with 1% glutamine, 0.5% penicillin/streptomycin and 5% fetal bovine serum. After seeding (24 h), HT-29 cells were treated, in quadruplicate, with increasing concentrations of the vegetable extracts, using ranges of concentrations 0.001–10 geq ww/ml and incubated for 24 h. The cytotoxicity assay was performed by adding a small amount of the CellTiter96^R AQueous One Solution Cell Proliferation Assay directly to culture wells, incubating for 4 h and then recording the absorbance at 450 nm with a ninety-six-well plate reader (MULTISKAN EX; Thermo Electron Corporation, Vantaa, Finland).

Genotoxicity studies

DNA damage was measured using single-cell gel electrophoresis (Comet assay). The alkaline version (pH > 13) of the assay was performed to detect single-strand breaks and alkali-labile sites, such as apyrimidinic and apurinic sites that are formed when bases are lost and oxidised.

The cells were seeded at 1×10^5 /ml in six-well plates in DMEM supplemented with 1% glutamine, 0.5% penicillin/streptomycin and 10% fetal bovine serum. After seeding (24 h), the HT-29 cells were treated with the vegetable

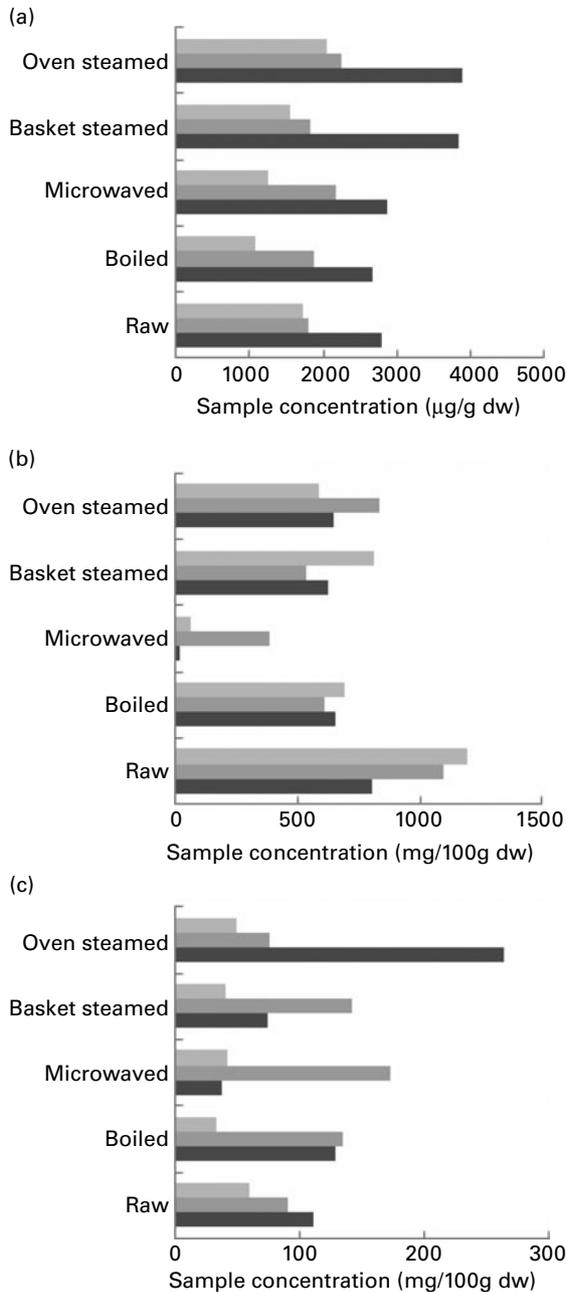


Fig. 1. Phytochemical compounds of raw and cooked *Brassica* specimens, values are expressed as mg/100 g of dry weight (dw; modified from Pellegrini *et al.*⁽²⁷⁾). (a) Glucosinolates; (b) ascorbic acid and (c) phenol compounds. ■, Cauliflower; ■, Brussels sprouts; ■, broccoli.

extracts, using the higher non-toxic concentrations as detected by the MTS assay for the uncooked vegetables. After incubation, the cells were trypsinised and re-suspended in DMEM supplemented with 1% glutamine, 0.5% penicillin/streptomycin and 10% fetal bovine serum at a concentration of 5×10^4 cell/ml.

After this, the suspensions were centrifuged (1 min, 800 g) to recover the cells. The cell pellets were re-suspended in 90 µl low melting agarose 0.7%, transferred onto degreased microscope slides previously dipped in 1% normal melting agarose for the first layer. The agarose was allowed to set for 15 min at 4°C before the addition of a final layer of low melting agarose. Cell lysis was carried out at 4°C overnight by exposing the cells to a buffer containing 2.5 M-NaCl, 100 mM-Na₂EDTA, 8 mM-Tris-HCl, 1% Triton X-100 and 10% dimethylsulfoxide, pH 10.

In an alkaline buffer, pH > 13, (1 mM-Na₂EDTA, 300 mM-NaOH, 0°C) the electrophoretic migration was performed (DNA unwinding: 20 min; electrophoresis: 20 min, 0.78 V/cm, 300 mA).

DNA was stained with 75 µl ethidium bromide (10 µg/ml) before examination at a 400× magnification under a Leica DMLS fluorescence microscope (excitation filter BP 515-560 nm, barrier filter LP 580 nm), using an automatic image analysis system (Comet Assay III; Perceptive Instruments Limited, Haverhill, Suffolk, UK).

The total percentage of fluorescence in the tail (TI, tail intensity) provided representative data on genotoxic effects. For each sample, coded and evaluated blind, 100 cells were analysed and the median value of TI was calculated. At least three independent experiments were performed for each extract and the mean of the median TI values was used for statistical analyses.

Antioxidant activity

The Comet assay was used to study the antioxidant effect of the test extracts. In that case, the amount of DNA damage caused by an oxidative damage-inducing agent on the cells pre-treated with the vegetable extracts was evaluated.

The cells were seeded at 1×10^5 /ml in six-well plates in DMEM supplemented with 1% glutamine, 0.5% penicillin/streptomycin and 10% fetal bovine serum. After seeding (24 h), the HT-29 cells were incubated with the vegetable extracts, using the higher non-toxic concentrations as detected by the MTS assay for the fresh vegetables, or with a known antioxidant agent, vitamin C (1 mM), and incubated for 24 h. After incubation, the cells were trypsinised and re-suspended in DMEM supplemented with 1% glutamine, 0.5% penicillin/streptomycin and 10% fetal bovine serum at a concentration 1×10^5 cell/ml for a further treatment in suspension, before performing the Comet assay, with H₂O₂ (100 µM) on ice for 5 min. After this, the suspensions were centrifuged twice (1 min, 800 g) to wash and recover the cells. The slides were prepared and analysed as reported above.

To better compare the ability of the extracts in reducing the DNA damage induced by H₂O₂ treatment, a TI variation

percentage was calculated as follows:

$$TI_{\text{variation}} (\%) = (TI_{\text{sample}+H_2O_2} / TI_{H_2O_2}) \times 100.$$

Statistical evaluation

The data were analysed using the statistical and graphical functions of SPSS 15 (SPSS, Inc., Chicago, IL, USA). Differences were assessed using ANOVA, followed by Bonferroni's post test as appropriate, for parameters normally distributed such as means of optical density values and of median TI values. In particular, for the Comet assay, statistical differences between controls and treated samples were first determined with the non-parametric Wilcoxon rank-sum test for each experiment. Significance was accepted at $P < 0.05$ level.

Results

Anti-proliferative activity

Anti-proliferative activity was detected by the MTS assay, a colorimetric method for determining the number of viable cells in proliferation (Fig. 2). In the range of the doses used ($10^{-3}/10^1$ g eq ww/ml), the raw vegetable extracts that were analysed showed high anti-proliferative/toxic activity on the HT-29 colon carcinoma cells. The lowest effective dose (LED, $P < 0.001$) was 0.25 g eq ww/ml for broccoli and 0.50 g eq ww/ml for cauliflower and Brussels sprouts.

The strong anti-proliferative activity detected with the raw broccoli extract was maintained with the microwave cooking method (LED: 0.25 g eq ww/ml, $P < 0.001$), while the boiled and steamed broccoli extracts showed no cytotoxic activity up to 4.00 g eq ww/ml.

All the cooking methods, except basket steaming up to 4.00 g eq ww/ml, partially maintained the anti-proliferative activity detected in the raw cauliflower extract: microwaving LED: 1.00 g eq ww/ml ($P < 0.01$); boiling LED: 2.00 g eq ww/ml ($P < 0.01$); oven steaming LED: 1.00 g eq ww/ml ($P < 0.05$).

In the case of Brussels sprouts, all the cooking methods applied decreased the anti-proliferative activity of the raw extract and no cytotoxic activity was traceable up to 4 g eq ww/ml.

Genotoxicity

On the basis of MTS values of raw and cooked vegetables we determined the sub-toxic concentrations to adopt for the DNA migration analysis (alkaline Comet assay) on HT-29 cells. To better compare the results between the extracts from fresh and cooked vegetables, which presented different antiproliferative activity, we analysed the genotoxicity induced by the highest non-toxic concentration identified with the raw extracts for all samples (i.e. broccoli: 0.01 g eq ww/ml; cauliflower and Brussels sprouts: 0.10 g eq ww/ml) and a concentration ten times higher only for cooked samples (broccoli: 0.10 g eq ww/ml; cauliflower and Brussels sprouts: 1.00 g eq ww/ml).

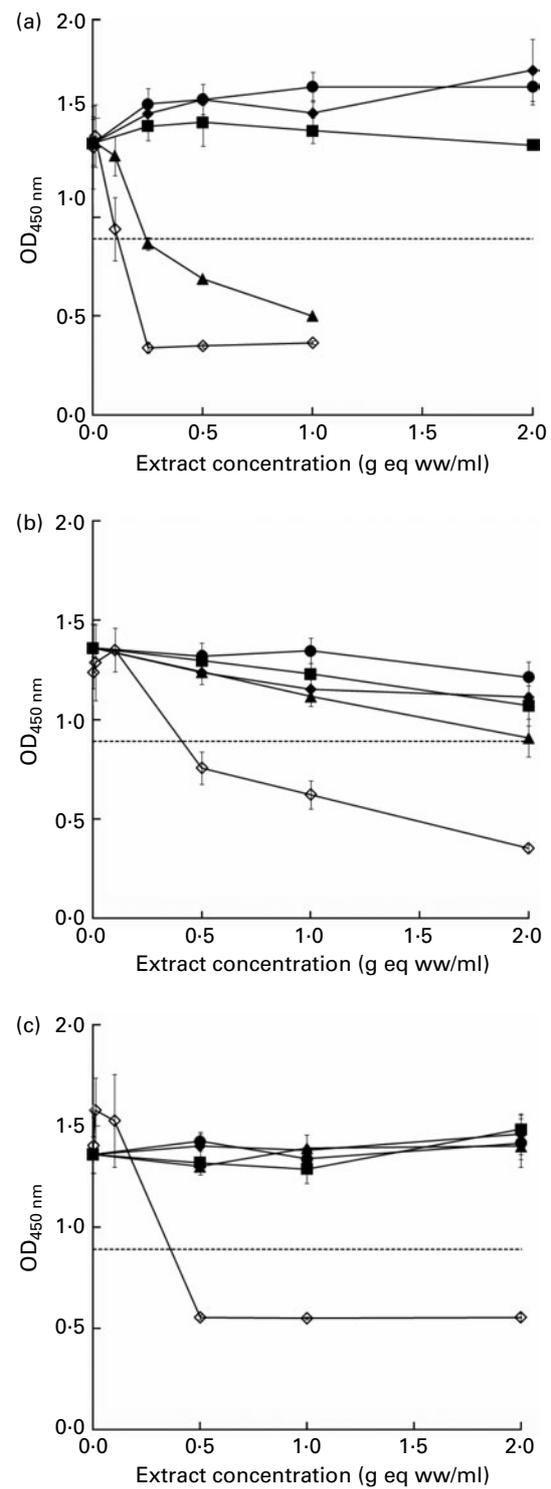


Fig. 2. Antiproliferative effects, detected by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, induced by increasing concentrations (equivalent of wet weight g/ml; g eq ww/ml) of raw and cooked (a) broccoli; (b) cauliflower; (c) Brussels sprouts extracts (24 h-treatment) on HT-29 cells. OD, optical density. \diamond —, Raw; \blacksquare —, boiled; \blacktriangle —, microwaved; \bullet —, basket steamed; \blacklozenge —, oven steamed; , cell seeding.

None of the vegetable extracts was able to induce DNA damage at the concentrations tested (Table 1).

Antioxidant activity

The cells were treated for 24 h with vegetable extracts, after discharging the medium plus the exhausted extract; they were subjected to 5 min shock with H_2O_2 (one of the most common oxidative damage-inducing agents). The ability to reduce the DNA damage induced by H_2O_2 treatment in HT-29 cells was chosen as an indicator of the chemopreventive capability of the vegetable extracts. Among the antioxidants, ascorbic acid (vitamin C) plays a central role as it contributes to the regeneration of vitamin E and constitutes a strong line of defence in retarding free radical-induced cellular damage⁽³⁰⁾. The antioxidant activity of ascorbic acid (1 mM) was determined. Vitamin C pre-treatment produced a significantly strong decrease (approximately 50%) of DNA damage induced by H_2O_2 at 100 μM ($P < 0.05$). All the raw vegetable extracts had significant antioxidant activity ($P < 0.05$) comparable with vitamin C, in particular the raw broccoli

Table 1. Genotoxicity evaluated by the Comet assay on HT-29 cells treated (24 h) with raw and cooked vegetable extracts at different concentrations* (Mean values and standard deviations)

Vegetable extract	Cooking method	Extract concentration (g eq ww/ml)	TI (%)		
			Mean	SD	
Untreated cells	—	—	0.31	0.20	
H_2O_2 (100 μM)	—	—	6.45	2.89	
Vitamin C (1 mM)	—	—	0.30	0.12	
Broccoli	Raw	0.01	0.22	0.12	
		0.01	0.37	0.30	
	Microwaved	0.10	0.31	0.22	
		0.01	0.27	0.08	
		0.10	0.18	0.02	
Basket steamed	0.01	0.22	0.03		
	0.10	0.35	0.21		
	0.01	0.12	0.04		
Oven steamed	0.10	0.34	0.24		
	Cauliflower	Raw	0.10	0.49	0.24
			0.10	0.50	0.18
Microwaved		1.00	0.33	0.17	
		0.10	0.58	0.08	
		1.00	0.36	0.15	
Basket steamed	0.10	0.35	0.10		
	1.00	0.30	0.15		
	0.10	0.38	0.05		
Oven steamed	1.00	0.25	0.08		
	Brussels sprouts	Raw	0.10	0.27	0.20
			0.10	0.43	0.35
Microwaved		1.00	0.40	0.01	
		0.10	0.46	0.20	
		0.10	0.19	0.11	
Basket steamed	0.10	0.36	0.24		
	1.00	0.26	0.09		
	0.10	0.25	0.14		
Oven steamed	1.00	0.40	0.07		

TI, tail intensity; g eq ww/ml, equivalent of wet weight g/ml. *Negative controls: HT-29 untreated and vitamin C treated (1 mM); positive control: H_2O_2 (100 μM).

extract showed its defensive capability at a ten-fold lower concentration than the other two raw vegetable extracts (Fig. 3).

Cooking broccoli with microwaving and steaming methods determined a slight lowering of the antioxidative properties

shown by the raw vegetable. In fact, the same significant DNA migration reduction ($P < 0.05$) induced by the raw extract was detected for cooked vegetables extracts at a concentration that was ten-fold higher. Otherwise, no antioxidant activity

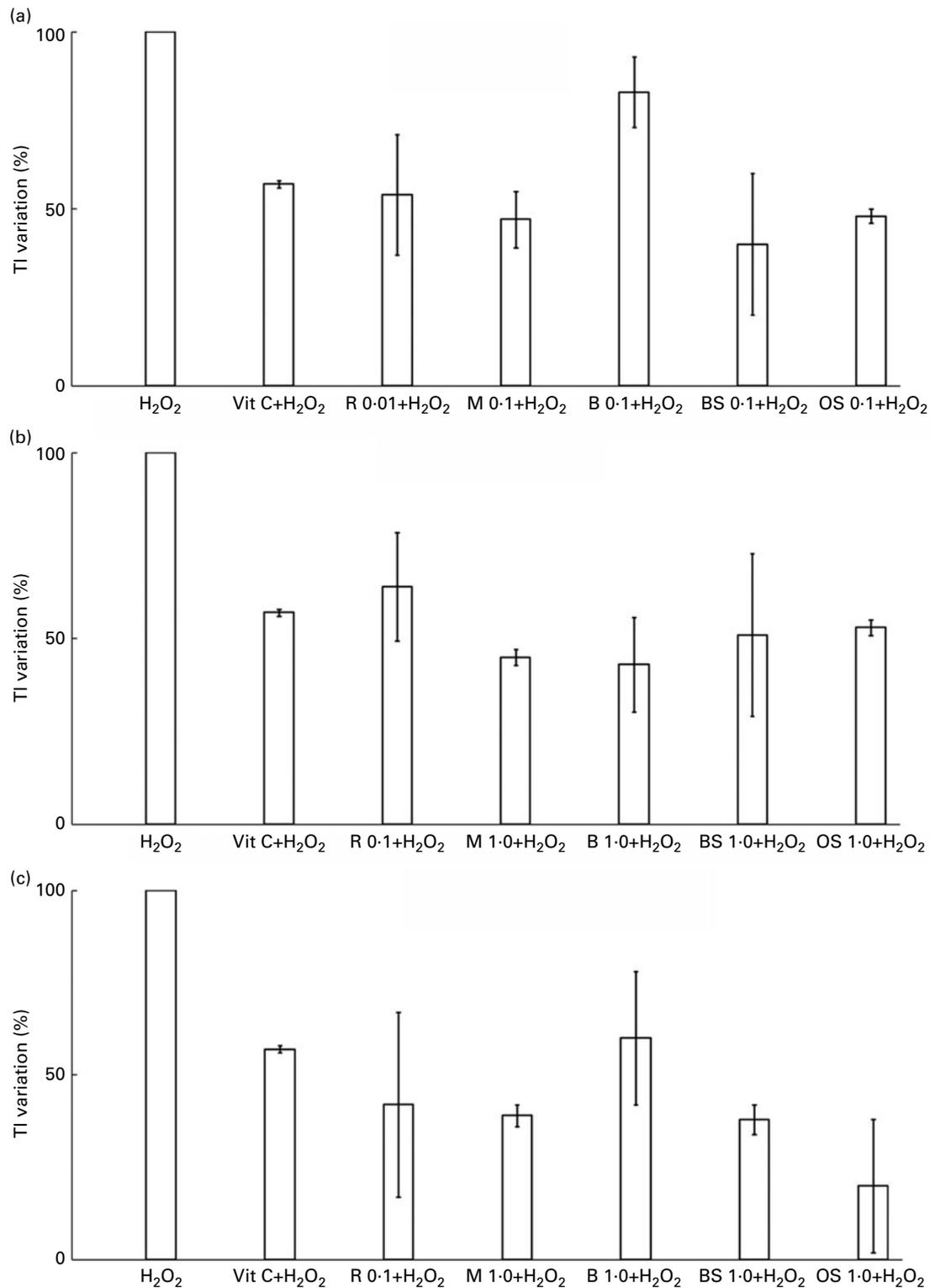


Fig. 3. Antioxidant activity evaluated by Comet assay on HT-29 cells treated (24h) with vitamin C (Vit C; 1 mM) or raw (R) or cooked vegetables extracts at different concentrations (0.01, 0.1, 1.0 g eq wet weight/ml) and treated with H₂O₂ (100 μM, 5 min). (a) Broccoli; (b) Cauliflower; (c) Brussels sprouts. It is reported the tail intensity (TI) variation (%): $(TI_{\text{sample}+H_2O_2} / TI_{H_2O_2}) \times 100$. M, microwaved; B, boiled; BS, basket steamed; OS, oven steamed.

was detected in the boiled broccoli at the extract concentrations tested (Fig. 3), in agreement with literature data⁽³¹⁾.

A significant antioxidant activity ($P < 0.05$) was detected in the cauliflower extracts, independently from the cooking method performed (Fig. 3). In particular, the microwaved vegetable extract seems more effective than the raw extract and vitamin C.

The microwaved Brussels sprouts extract showed an antioxidant activity comparable with the raw extract and vitamin C, whereas the extracts of boiled and steamed Brussels sprouts showed the same protective activity at ten-fold higher concentrations (Fig. 3).

Discussion

In this study, the effect of cooking practices on the anti-proliferative and/or antioxidant properties of three fresh *Brassica* species (broccoli, cauliflower and Brussels sprouts) was evaluated by MTS and Comet assays. Data demonstrated a significant dose-dependent anti-proliferative effect when the human colon cancer cells (HT-29) were treated with uncooked vegetable extracts. The highest anti-proliferative activity was found with the broccoli extract with the LED twofold lower than the Brussels sprouts and cauliflowers extracts. This anti-proliferative rank order could be linked to the higher content of bioactive compounds, mainly glucosinolates, present in broccoli than in the other *Brassica* vegetables analysed (Fig. 1)⁽²⁷⁾, even though a difference in single compounds present cannot be ruled out. It is known that some chemopreventive vegetable components could induce cell cycle arrest in HT-29 cells; in particular the hydrolysis of glucosinolates by myrosinase could induce the formation of chemopreventive molecules such as isothiocyanates^(32,33). Even though we did not measure the myrosinase activity in the extracts, during their preparation the Stomaker treatment could have mimicked the mastication process, determining the activation of myrosinase and, hence, the formation of hydrolysis products, as during the *Brassica* processing before cooking (i.e. chopping, treading, etc.)⁽³⁴⁾. Among the vegetables analysed, broccoli has the highest concentration of the glucosinolate glucoraphanin, which could be hydrolysed by myrosinase in the reactive isothiocyanate sulforaphane with chemopreventive properties. Sulforaphane exerts its anti-proliferative effect by arresting the cell cycle; this arrest has been documented in the colon, prostate, breast, bladder and T cells⁽³³⁾.

The release of active molecules from the Brassicaceae tissues, following the glucosinolate hydrolysis by myrosinase, is strongly influenced by the cooking practice adopted⁽³⁴⁾. The lowering or disappearance of the *Brassica* anti-proliferative effects detected after cooking practices, particularly boiling and steaming, could be ascribed to an alteration of the glucosinolate–myrosinase system⁽³⁵⁾. Conversely, the higher retention of the anti-proliferative effect of microwaved *Brassica* samples with respect to other cooking practices could be linked to a reduced inactivation of myrosinase due to the mild cooking conditions applied (i.e. low energy

inputs with consequent low temperature cooking), as demonstrated by Verkerk & Dekker⁽³⁶⁾.

Many *in vitro* and *in vivo* studies demonstrated that some molecules (i.e. polyphenols, catechins, etc.) present in food are anti-carcinogenic by inducing apoptosis and inhibiting cell growth^(10,22). Probable mechanisms of action include antioxidant and free-radical scavenging activity. On the other hand, vegetables, including *Brassica*, present a variable chemical complex mixture and could also contain compounds with genotoxic activity^(37,38). The vegetable extracts, evaluated in this research, were analysed both for their genotoxic or anti-genotoxic (antioxidant) activities by the Comet assay. DNA migration analysis of HT-29 cells treated (24 h) with vegetable extracts alone, at sub-toxic concentrations determined on MTS values, did not show any induced DNA damage, whereas HT-29 cells pre-treated for 24 h with raw and cooked *Brassica* and treated with H₂O₂ (100 μM, 5 min at 0°C) showed different sensitivity to DNA oxidative damage, as revealed by the alkaline Comet assay.

The chemopreventive action that was detected was determined by an endogenous response, due to the fact that the vegetable extract was removed before H₂O₂ treatment. In particular, raw, microwaved, boiled (except broccoli) and steamed vegetable extracts, presented a protective antioxidative action comparable with vitamin C (1 mM), as demonstrated by the reduced DNA migration. It is noteworthy that only the cooked cauliflower extracts showed an antioxidant activity comparable to the raw extract (0.10 g eq ww/ml) with the same TI (%) variation coefficient.

Among the different cooking practices, the boiling treatment seems to determine a different antioxidant status depending on the kind of *Brassica* analysed; although the highest loss of activity was found with broccoli, their cooked extracts remained the more active. The steaming and microwaving practices showed a general good retention of the antioxidant activity. Based on the present results, the antioxidant activity of raw extracts could be probably ascribed to the glucosinolate hydrolysis products. On the contrary, the antioxidant activity of cooked *Brassica* vegetables could be due to the formation of new compounds following the cooking, for instance, polyphenols with a different oxidation state that could exhibit their antioxidant activity not being masked by the glucosinolates hydrolysis products. However, these hypotheses should be confirmed by the identification and quantification of phytochemical compounds present in the aqueous extracts as no clear relationships were found when data on biological activities were compared with the concentration of chemical compounds present in the vegetables, before the extraction process.

At a first glance, the comparative analysis of the raw extract toxicological data (anti-proliferative and antioxidant activities) seems to suggest that high anti-proliferative activity could be concomitant with high antioxidant activity, suggesting that the chemopreventive action could be related to the control of the redox cellular status. This correlation disappears when data of cooked vegetable extracts are observed; generally, the loss of anti-proliferative activity seems independent of the antioxidant activity, suggesting a more complex design

involving several pathways differently regulated by the complex mixtures present in the extracts. These results are in agreement with Boivin *et al.*⁽³⁹⁾, who found that the anti-proliferative effect of *Brassica* vegetables, among others, largely independent of their antioxidant properties evaluated in *in vitro* experiments.

In conclusion, the analyses of the present data on the human colon carcinoma cell line HT-29 indicate that *Brassica* extracts could positively alter cellular endogenous chemoprotective status.

The lack of a chemical characterisation of the water extracts prevents us from a clear correlation among the biological activities tested and the specific compounds. Nevertheless, the increased ability of cells, treated with the *Brassica* samples, to contrast the reactive oxygen species damaging activity seems to be related to the complex mixture of the protective substances present in the vegetables more than to a single class of molecules.

The cooking methods applied influence the anti-proliferative activity of the *Brassica* extracts but do not alter considerably the high antioxidant activity presented by the raw vegetables. These data provide a new insight into the influence of domestic treatment on the quality of food, which could support the recent epidemiological studies suggesting that the consumption of cruciferous vegetables, mainly cooked food, may be related to a reduced risk of developing cancer.

Acknowledgements

This study was supported by Regione Emilia Romagna; SIQUAL project (Laboratorio Regionale per la Sicurezza e la Qualità degli Alimenti). A. B., N. P., and C. R. designed the research, L. F., T. M., C. M., S. G., and F. M. conducted the research, A. B., L. F. and N. P. analysed the data, A. B. and N. P. wrote the paper, C. R. and C. M. provided critical revision of the manuscript, A. B. had primary responsibility for the final content. All authors read and approved the final manuscript. We thank Antonietta Girasuolo for excellent technical assistance. La Linea Verde spa, Insalate Pronte (Manerbio, Brescia, Italy), is acknowledged for the gift of *Brassica* vegetables. There are no conflicts of interest.

References

- Mukherjee S, Gangopadhyay H & Das DK (2008) Broccoli: a unique vegetable that protects mammalian hearts through the redox cycling of the thioredoxin superfamily. *J Agric Food Chem* **56**, 609–617.
- Kim MK & Park JH (2009) Conference on “Multidisciplinary approaches to nutritional problems”. Symposium on “Nutrition and health”. Cruciferous vegetable intake and the risk of human cancer: epidemiological evidence. *Proc Nutr Soc* **68**, 103–110.
- Lynn A, Collins A, Fuller Z, *et al.* (2006) Cruciferous vegetables and colo-rectal cancer. *Proc Nutr Soc* **65**, 135–144.
- Turkmen N, Sari F & Velioglu YS (2005) The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chem* **93**, 713–718.
- Podsedek A, Sosnowska D, Redzynia M, *et al.* (2006) Antioxidant capacity and content of *Brassica oleracea* dietary antioxidants. *Int J Food Sci Technol* **41**, 49–58.
- Podsedek A (2007) Natural antioxidants and antioxidant capacity of *Brassica* vegetables: a review. *LWT-Food Sci Technol* **40**, 1–11.
- Higdon JV, Delage B, Williams DE, *et al.* (2007) Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. *Pharmacol Res* **55**, 224–236.
- Vallejo F, Tomás-Barberán FA & García-Viguera C (2002) Glucosinolates and vitamin C content in edible parts of broccoli florets after domestic cooking. *Eur Food Res Technol* **215**, 310–316.
- Song LJ & Thornalley PJ (2007) Effect of storage, processing and cooking on glucosinolate content of *Brassica* vegetables. *Food Chem Toxicol* **45**, 216–224.
- Surh YJ (2003) Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* **3**, 768–780.
- Berger MM (2005) Can oxidative damage be treated nutritionally? *Clin Nutr* **24**, 172–183.
- Nicoli MC, Anese M & Parpinel M (1999) Influence of processing on the antioxidant properties of fruit and vegetables. *Trends Food Sci Technol* **10**, 94–100.
- Lee SK & Kader AA (2000) Preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharv Biol Technol* **20**, 207–220.
- Ou B, Huang D, Hampsch-Woodill M, *et al.* (2002) Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity ORAC and ferric reducing antioxidant power FRAP assays. A comparative study. *J Agric Food Chem* **50**, 3122–3128.
- Bernhardt S & Schlich E (2006) Impact of different cooking methods on food quality: retention of lipophilic vitamins in fresh and frozen vegetables. *J Food Eng* **77**, 327–333.
- Miglio C, Chiavaro E, Visconti A, *et al.* (2008) Effects of different cooking methods on nutritional and physicochemical characteristics of selected vegetables. *J Agric Food Chem* **56**, 139–147.
- Link LB & Potter JD (2004) Raw versus cooked vegetables and cancer risk. *Cancer Epidemiol Biomarkers Prev* **13**, 1422–1435.
- Dewanto V, Wu X, Adom KK, *et al.* (2002) Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J Agric Food Chem* **50**, 3010–3014.
- Bugianesi R, Salucci M, Leonardi C, *et al.* (2004) Effect of domestic cooking on human bioavailability of naringenin, chlorogenic acid, lycopene and β -carotene in cherry tomatoes. *Eur J Nutr* **43**, 360–366.
- Lo Scalzo R, Fibiani M, Mennella G, *et al.* (2010) Thermal treatment of eggplant (*Solanum melongena* L.) increases the antioxidant content and the inhibitory effect on human neutrophil burst. *J Agric Food Chem* **58**, 3371–3379.
- Heo BG, Chon SU, Park YJ, *et al.* (2009) Antiproliferative activity of Korean wild vegetables on different human tumor cell lines. *Plant Foods Hum Nutr* **64**, 257–263.
- Sharif T, Auger C, Alhosin M, *et al.* (2010) Red wine polyphenols cause growth inhibition and apoptosis in acute lymphoblastic leukaemia cells by inducing a redox-sensitive up-regulation of p73 and down-regulation of UHRF1. *Eur J Cancer* **46**, 983–994.
- Cemeli E, Baumgartner A & Anderson D (2009) Antioxidants and the Comet assay. *Mutat Res* **681**, 51–67.

24. Wasson GR, McKelvey-Martin VJ & Downes CS (2008) The use of the comet assay in the study of human nutrition and cancer. *Mutagenesis* **23**, 153–162.
25. Wong VWC, Szeto YT, Collins AR, *et al.* (2005) The Comet assay: a biomonitoring tool for nutraceutical research. *Curr Topics Nutraceutical Res* **3**, 1–14.
26. Riso P, Martini D, Visioli F, *et al.* (2009) Effect of broccoli intake on markers related to oxidative stress and cancer risk in healthy smokers and nonsmokers. *Nutr Cancer* **61**, 232–237.
27. Pellegrini N, Chiavaro E, Gardana C, *et al.* (2010) Effect of different cooking methods on color, phytochemical concentration and antioxidant capacity of raw and frozen *Brassica* vegetables. *J Agric Food Chem* **58**, 4310–4321.
28. Laky B, Knasmüller S, Gminski R, *et al.* (2002) Protective effects of Brussels sprouts towards B[a]p-induced DNA damage: a model study with the single-cell gel electrophoresis (SCGE)/Hep G2 assay. *Food Chem Toxicol* **40**, 1077–1083.
29. Kassie F, Laky B, Gminski R, *et al.* (2003) Effects of garden and water cress juices and their constituents, benzyl and phenethyl isothiocyanates, towards benzo(a)pyrene-induced DNA damage: a model study with the single cell gel electrophoresis/Hep G2 assay. *Chem Biol Interact* **142**, 285–296.
30. Chan AC (1993) Partners in defense, vitamin E and vitamin C. *Can J Physiol Pharmacol* **71**, 725–731.
31. Knutson KM, Marth EH & Wagner MK (1987) Microwave heating of food. *Lebensm-Wiss Technol* **20**, 101–110.
32. Keum Y-S, Jeong W-S, Kong AN, *et al.* (2004) Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. *Mutat Res* **555**, 191–202.
33. Juge N, Mithen RF & Traka M (2007) Molecular basis for chemoprevention by sulforaphane: a comprehensive review. *Cell Mol Life Sci* **64**, 1105–1127.
34. Rungapamestry V, Duncan AJ, Fuller Z, *et al.* (2007) Effect of cooking *Brassica* vegetables on the subsequent hydrolysis and metabolic fate of glucosinolates. *Proc Nutr Soc* **66**, 69–81.
35. Dekker M, Verkerk R & Jongen WMF (2000) Predictive modelling of health aspects in the food production chain: a case study on glucosinolates in cabbage. *Trends Food Sci Technol* **11**, 174–181.
36. Verkerk R & Dekker M (2004) Glucosinolates and myrosinase activity in red cabbage (*Brassica oleracea* L. var. *Capitata f. rubra* DC.) after various microwave treatments. *J Agric Food Chem* **52**, 7318–7323.
37. Kassie F, Parzefall W, Musk S, *et al.* (1996) Genotoxic effects of crude juices from *Brassica* vegetables and juices and extracts from phytopharmaceutical preparations and spices of cruciferous plants origin in bacterial and mammalian cells. *Chem Biol Interact* **102**, 1–16.
38. Heres-Pulido ME, Dueñas-García I, Castañeda-Partida L, *et al.* (2010) Genotoxicity studies of organically grown broccoli (*Brassica oleracea* var. *italica*) and its interactions with urethane, methyl methanesulfonate and 4-nitroquinoline-1-oxide genotoxicity in the wing spot test of *Drosophila melanogaster*. *Food Chem Toxicol* **48**, 120–128.
39. Boivin D, Lamy S, Lord-Dufour S, *et al.* (2009) Antiproliferative and antioxidant activities of common vegetables: a comparative study. *Food Chem* **112**, 374–380.