JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 5227–5234 5227
DOI:10.1021/jf9002973

# Analysis of Phytochemical Composition and Chemoprotective Capacity of Rocket (*Eruca sativa* and *Diplotaxis tenuifolia*) Leafy Salad Following Cultivation in Different Environments

Jing Jin,[†] Olga A. Koroleva,[‡] Trevor Gibson,[§] June Swanston,[†] Jane Magan,[†] Yan Zhang,[†] Ian R. Rowland,[†] and Carol Wagstaff*,[†]

[†]Department of Food Biosciences, [‡]School of Biological Sciences, [§]The BioCentre, and University of Reading, Whiteknights, Reading RG6 6AP, U.K.

Consumption of green leafy vegetables is associated with reduced risk of several types of cancer and cardiovascular disease. These beneficial effects are attributed to a range of phytochemicals including flavonoids and glucosinolates, both of which are found in high levels in Brassicaceous crops. Rocket is the general name attributed to cultivars of *Eruca sativa* and *Diplotaxis tenufolia*, known as salad rocket and wild rocket, respectively. We have shown that different light levels during the cultivation period of these crops have a significant impact on the levels of flavonoids present in the crop at harvest, with over 15-fold increase achieved in quercetin, isorhamnetin, and cyanidin in high light conditions. Postharvest storage further affects the levels of both flavonoids and glucosinolates, with cyanidin increasing during shelf life and some glucosinolates, such as glucoiberverin, being reduced over the same storage period. In vitro assays using human colon cell lines demonstrate that glucosinolate-rich extracts of *Eruca sativa* cv. Sky, but not *Diplotaxis tenufolia* cv. Voyager, confer significant resistance to oxidative stress on the cells, which is indicative of the chemoprotective properties of the leaves from this species. Our findings indicate that both pre and postharvest environment and genotypic selection, when developing new lines of Brassicaceous vegetables, are important considerations with the goal of improving human nutrition and health.

**KEYWORDS: Rocket; *Eruca sativa*; *Diplotaxis tenufolia*; flavonoids; glucosinolates; isothiocyanates; chemoprotective**

## INTRODUCTION

The Brassicaceae is a large family of plants, and epidemiological evidence has shown that consumption of *Brassica* vegetables is associated with a highly significant reduction of cancer risk (*1*). In these studies, researchers have been focusing on major vegetable crops including Brussels sprouts, cabbage, broccoli, watercress, and cauliflower. These vegetables are rich sources of bioactive compounds with chemoprotective properties including glucosinolates, flavonoids, fiber, folate, and carotenoids. Glucosinolates (GSL) are sulfur-containing phytonutrients responsible for the sharp taste of cruciferous vegetables and are primarily synthesized by the plant as part of a defense mechanism against insect predators (*2*). It has been reported that their distribution among cruciferous vegetables varies, depending on the species, the plant organ and age, environmental factors, and other agricultural conditions (*3*). Within the plant cell, glucosinolates present in the vacuole are spatially separated from the hydrolyzing enzyme myrosinase in the cytoplasm. Glucosinolate hydrolysis by myrosinase, upon plant injury or during food processing, results in the formation of biologically active compounds, including indoles and isothiocyanates (ITC) reported to be involved in anticarcinogenic processes (*2*).

Salad species in the Brassicaceae family such as *Eruca sativa* (salad rocket) and *Diplotaxis tenuifolia* (wild rocket) have been grown in the Mediterranean area since Roman times and now have been cultivated in various places for their use in salads and soups. They have a rich, peppery taste and are exceptionally strongly flavored for a leafy green crop. Rocket has been reported to contain high levels of vitamin C, glucosinolates, flavonoids, and phenolics (*4*). These compounds are all susceptible to environmental conditions before and after harvest, which affects the qualitative and quantitative profile of phytonutrients in the leaf (*5*). Flavonoids are a group of compounds that encompass a range of phytonutrients that have an antioxidant function. Although humans benefit from the consumption of plants with high antioxidant capacity, they have multiple functions in the plant related to quenching reactive oxygen species, such as protecting the photosynthetic machinery, as defense against chewing insects, and as part of the hypersensitive response to pathogen attack (*6*).

Recently, the major glucosinolate in the leaves of salad rocket was identified as 4-mercaptobutylglucosinolate (glucosativin) (*7*), which upon hydrolysis produces 4-mercaptobutyl-isothiocyanate (sativin). This glucosinolate and its isothiocyanate may be related

*To whom correspondence should be addressed. E-mail: c.wagstaff@reading.ac.uk.

 Published on Web 06/02/2009 

to the distinct pungent or bitter taste and flavor of salad rocket. Ontogenic LC/MS profiling of glucosinolates in a range of rocket species confirmed that glucosativin is predominantly present in *Diplotaxis erucoides* and *Bunias orientalis* (Turkish rocket) (*7*). Other glucosinolates have been found in rocket, including 4-(β-D-glucopyranosyldisulfanyl)butyl glucosinolate, 4-methylthiobutylglucosinolate (glucoerucin), and glucoraphanin (*8*). Experiments using pure glucosinolate or isothiocyanate compounds showed that both can exert either genotoxic or antigenotoxic effects depending on the type of cell lines and the doses used (*9*). However, preliminary quinine reductase activity data using rodents suggest that the isothiocyanate sulforaphane (SF), derived from glucoraphanin, is more beneficial when present in whole broccoli than as purified SF (*10*), providing strong evidence that it is preferable to analyze the impact of whole foods on human health and thereby enabling the interaction of the phytonutrient profile in that food to be assessed, rather than using individual pure compounds.

All rocket tissues, except roots, in the species studied to date contain significant levels of polyglycosylated flavonoids, and the core aglycones are kaempferol, quercetin, and isorhamnetin (*7*). Metabolite profiling of flavonoids revealed that *Eruca vesicaria* leaves contained kaempferol derivatives as principal compounds, whereas *Diplotaxis tenuifolia* instead accumulated quercetin derivatives (*11*). In the past two decades, plant-derived phenolics and flavonoids have garnered great interest as they are known to have direct antioxidant and free-radical scavenging activities, but can also induce the expression of various genes encoding metabolic enzymes thought to decrease the risk of various diseases and disorders, including cancer, cardiovascular disease, and immune dysfunctions (*12*).

In this study, we investigated the impact of controlled preharvest stress, induced by altering light intensity, on the qualitative and quantitative profile of key dietary phytonutrients in *Eruca sativa* cv. Sky (salad rocket) and *Diplotaxis tenufolia* cv. Voyager (wild rocket). In addition, the effect on the expression of genes involved in glucosinolate metabolism and on antigenotoxicity of rocket leaf extracts was studied.

## MATERIALS AND METHODS

**Plant Material.** Seeds of *Eruca sativa* cv. Sky (salad rocket) and *Diplotaxis tenuifolia* cv. Voyager (wild rocket) were obtained from Nicky's Seeds (Broadstairs, Kent, U.K.). The seeds were germinated under the same conditions under a minimum temperature of 25 °C. After two weeks, the plants were placed randomly into the different light intensity treatment groups, low light intensity, 20−30 μmol $m^{-2}$ $s^{-1}$, and high light intensity, 80−120 μmol $m^{-2}$ $s^{-1}$, and were grown for another six weeks. The low light intensity treatment was achieved by shading the glass with netting. Leaves from 10 plants per cultivar per treatment group were harvested together and either placed in Ziploc-type freezer bags at 4 °C for postharvest storage or snap frozen directly in liquid nitrogen and ground using a pestle and mortar.

**Chemicals and Cell Lines.** All chemicals were obtained from Sigma (Poole, U.K.) unless otherwise stated. Human colonic cancer HT-29 cells were from European Collection of Cell Cultures (ECACC) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, nonessential amino acids, 10 U $mL^{-1}$ penicillin, and 10 μg $mL^{-1}$ streptomycin (all from Lonza, Basel, Switzerland) at 37 °C in a humidified incubator (5% $CO_2$). The medium was changed every two days.

**Glucosinolates Extraction and LC/MS Analysis.** Replicate samples of ground rocket leaves (3 × 40 mg) were extracted with 1.0 mL of 70% (v/v) methanol at 70 °C for 20 min. Immediately prior to LC-MS analysis, the extract was diluted 10-fold with HPLC grade water. To 1 mL of diluted extract, 5 μL of sinigrin (12 mM made up in 70% methanol) was added for a final concentration of 56 ng $\mu L^{-1}$ as an internal reference standard for quantification. For quantitative analysis, a 5-point calibration curve of diluted sinigrin standards was used. LC-MS/MS analysis was performed in negative mode on Esquire HCT ESI (Bruker) coupled to LC (Agilent) with a reversed phase Varian Pursuit 50 × 2.1 mm C18 column. The individual glucosinolates were separated during a 12 min chromatography run in acetonitrile 5−100% gradient and identified by their nominal mass, characteristic fragment ions, and sulfur isotopic signature. The amount of each glucosinolate in the sample was estimated as a corresponding peak elution area on the chromatogram and normalized by the internal sinigrin standard.

**Acid Hydrolysis Extraction of Flavonoids for HPLC Analysis of Aglycone Identification.** Between 70 and 90 mg of each sample was placed in a screw-cap tube with 5 mL of 2 M hydrochloric acid in HPLC grade methanol. Aluminium foil was wrapped around the tube to prevent deterioration of the flavonoids in light, and samples were placed on a roller/stirrer for 45 min before filtering through 0.45 μm filter discs into screw cap microcentrifuge tubes (roughly 1 mL in each tube). These were then placed in a dried heating block set to 85 °C for 60 min and left to cool for 30 min before being stored at −20 °C.

**Antioxidant Capacity.** The total antioxidant activity of the extracts was determined by using the Trolox-equivalent antioxidant capacity (TEAC) assay based on the oxidation of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) by potassium persulphate to form a stable radical ($ABTS^+$) and the direct scavenging of the preformed radical by rocket leaf extracts (*13*). Trolox (6-hydroxyl-2,5,7,8-tetramethychroman-2-carboxylic acid) was used as an antioxidant standard.

**Nucleic Acid Extraction and qRT-PCR.** DNA was extracted from leaf tissue by grinding in 2× Cetyl trimethylammonium bromide (CTAB) buffer containing β-mercaptoethanol prior to incubation at 60 °C for 20 min and the addition of phenol/chloroform/isoamylalcohol (25:24:1). Following centrifugation, DNA was precipitated with ethanol and recentrifuged to form a pellet, which was resuspended in Tris-EDTA at pH 7.8. RNA extraction, cDNA synthesis, and qRT-PCR were conducted as described in Wagstaff et al. (*14*). Partial genomic sequences were amplified using the following primers: ESPF, GTCATTTCTCCAGCCACAGG; ESPR, GCTTGCCGGCTCCTCCTCTT; ESM1F, CCCTGAGGGAAGTTCTCCAT; ESM1R, CCTTGCCGGGTTTGTATTC. Primers used to amplify cDNA for qRT-PCR were as follows: ESPF, GTCATTTCTCCAGCCACAGG; ESPR2, GTGGAAGCTACGAGGAGTGG; ESM1F, CCCTGAGGGAAGTTCTCCAT; ESM1R2, AAATTCGAACGCAACTAGCC. Cloning and sequencing of ESTs was performed according to Wagstaff et al. (*15*)

**Comet Assay.** The comet assay to assess DNA damage was performed as previously described by Gill et al. (*16*). HT-29 cells were treated with rocket leaf glucosinolate extracts (approximately 40 mg dry weight (DW) $mL^{-1}$) diluted with culture media to the concentrations of 0.1, 1, 10, or 100 μg DW $mL^{-1}$ or flavonoid extracts (approximately 16 mg DW $mL^{-1}$) diluted with culture media to the concentrations of 0.1, 1, or 10 μg DW $mL^{-1}$ for 24 h prior to exposure to hydrogen peroxide ($H_2O_2$). For glucosinolate extracts, 0.25% extract buffer (70% methanol) in media (v/v) was used as a negative control, and for flavonoid extracts, 0.0625% extract buffer (5 M HCl in methanol) in culture media (v/v) was used. $H_2O_2$ only treated cells were used as the positive control. Stained cells were imaged at 400× magnification on an Olympus BX51 Upright fluorescence microscope equipped with an Andor Luca EMCCD camera. Fifty cells were counted and analyzed using Komet 5.0 imaging and analysis software (Andor technology, Belfast, U.K.). The effect of myrosinase was tested using an in situ method, i.e., a myrosinase-catalyzed reaction was performed in a cell culture medium directly in contact with cells (*17*).

**Statistics.** Data shown represent the mean ± SEM. Unless otherwise stated, these means were calculated from the means of triplicate replicates obtained in 3 independent extracts. One-way ANOVA was used to determine significance of the experimental variables, and the significance of individual treatment groups in comparison to the controls was determined with the Fisher's least significance difference (LSD) analysis with a confidence interval of 95%.

## RESULTS AND DISCUSSION

**Cultivation of Salad and Wild Rocket Cultivars (*Eruca sativa* cv. Sky and *Diplotaxis tenuifolia* cv. Voyager, respectively) under Different Light Intensities Had a Profound Effect on Their Appearance and Physiology.** Plants grown under higher light had smaller

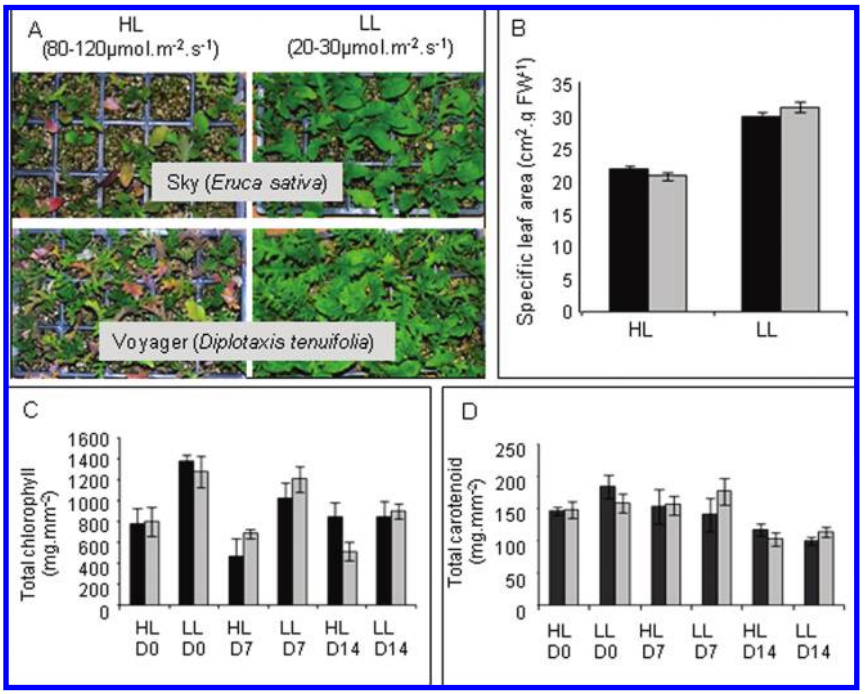


**Figure 1.** Rocket physiology following cultivation under different light conditions: (**A**) Appearance at time of harvest showing different leaf morphology and anthocyanin accumulation under high light intensity. (**B**) Specific leaf area of Sky (black bars) and Voyager (gray bars) showing increased leaf thickness in low light. (**C**) Total chlorophyll of Sky (black bars) and Voyager (gray bars) grown in high light (HL) or low light (LL) at harvest (D0) and after seven (D7) and 14 days (D14) of storage at 4 °C. (**D**) Total carotenoid content of Sky (black bars) and Voyager (gray bars) grown in high light (HL) or low light (LL) at harvest (D0) and after seven (D7) and 14 days (D14) of storage at 4 °C. $N = 3$.

leaves and appeared to accumulate anthocyanins (**Figure 1A**). Accumulation of anthocyanin pigments in plants is a common phenomenon related to a number of environmental stress stimuli including extremes of heat, light, and cold (*18*). The leaves of both cultivars also became thickened as a result of the low light environment, as is typical of shade leaves (*19*) (**Figure 1B**). Measurement of chlorophyll and carotenoid pigments showed that plants grown in low light accumulated more chlorophyll per unit area during the growth period, showing a similar response to shade adapted leaves in other species (*19*), although levels of carotenoids were unchanged. However, chlorophyll levels declined throughout shelf life in leaves from both cultivars grown under shade conditions (**Figure 1C**), indicating that these leaves senesced more readily once detached and stored in the dark at 4 °C. In contrast, carotenoid levels were more stable in both cultivars and conditions over the first week of storage, but leaves had lost approximately one-third of the harvested carotenoid content after two weeks of storage (**Figure 1D**). Carotenoids are well known to be stable during leaf senescence, as evidenced in all plants that display red/orange autumn leaf colors, and provide protection against harmful reactive oxygen species (*20*). Carotenoids, and by inference vitamin A, were also found by Bergquist et al. (*21*) to be stable, or even increase, in spinach leaves during postharvest storage.

Extracts were made from freshly harvested leaves of Sky and Voyager cultivars to specifically isolate total glucosinolates and flavonoids. The total antioxidant capacity of each extract was determined using the TEAC assay. The two cultivars were very similar to each other, and all of the extracts made from leaves grown in high light had a similar antioxidant capacity; however, leaves from plants grown in low light had a significantly lower antioxidant capacity (**Figure 2** and Table S1 (Supporting Information)). This is consistent with work on other species, which has shown that secondary metabolite production linked to antioxidants is increased by environmental stress conditions (*22*).

**Flavonoid Accumulation Is Correlated with Environmental Stress.** Flavonoid profiles were examined in more detail using quantitative HPLC against known standards. In common with previous studies of rocket cultivars (*11*), the predominant flavonoids were quercetin, kaempferol, and isorhamnetin (**Figure 3A−C** and Table S1 (Supporting Information)), and we also found considerable accumulation of cyanidin in high light (**Figure 3D** and Table S1 (Supporting Information)). The most striking feature of all of the flavonoids investigated was the difference in levels produced under the two different growth conditions with the high light environment leading to the production of up to 15-fold greater levels compared to that of the shade grown leaves. None of the flavonoids studied showed a significant change during shelf life from leaves grown under high light conditions, but kaempferol showed an increase during storage in leaves grown under low light, and this flavonoid was present in significantly greater amounts in Sky (salad) rocket compared to that in Voyager after both growth conditions. This supports our central hypothesis that many of these secondary metabolites of dietary importance are produced as a result of plant stress; hence, higher levels are found in plants grown under stressful conditions and when leaves have been stored in conditions that induce a cold stress response. An increase in total antioxidants and total phenolics was also observed during the course of the shelf life of rocket when the samples were maintained in controlled atmosphere at 4 °C (*4*); similarly, Vinã and Chaves (*22*) observed an increase in the antioxidant capacity of stored celery, which was accentuated by low temperatures. Cyanadin, the flavonoid linked to anthocyanin production, also showed a significant increase during shelf life in both cultivars, which we again hypothesize to be a cold stress response. The wild rocket cultivar Voyager showed much greater accumulation during shelf life in leaves from the high light environment, possibly indicating that this wild cultivar has retained its capacity to synthesize protective pigments when under stress to a greater extent than the

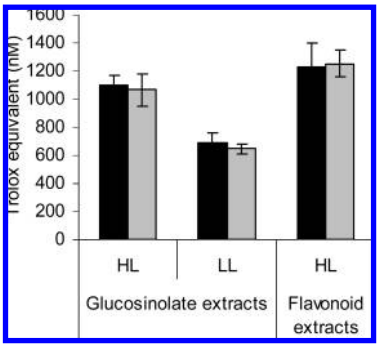


**Figure 2.** Antioxidant capacity of Sky (black bars) and Voyager (gray bars) rocket extracts (100 $\mu$g DW mL$^{-1}$) determined by TEAC. Relative antioxidant activities were calculated in Trolox equivalents (nM). $N = 3$.

more domesticated salad rocket, which has not been selected for survival strategies such as cold tolerance.

**Glucosinolate Profile Is Altered by Postharvest Storage.** Glucosinolate (GSL) profiling revealed that several compounds are common to both cultivars, but they were present in very different proportions at harvest and after 14 days storage at 4 °C (**Figure 4A−B** and Table S1 (Supporting Information)). The leaves of Sky and Voyager rocket consistently contained a high percentage of 4-mercaptobutyl-glucosinolate, in both the monomer and dimer forms (40−64%), as compared to the other glucosinolates, in common with the proportions found in sprouts of these species from diverse geographical locations by Bennett et al. (*7*). The glucosinolate 4-($\beta$-D-glucopyranosyldisulfanyl) butyl GSL was also identified in the leaves of both rocket varieties. The quantity of this glucosinolate is very similar in Sky and Voyager rocket leaves and was not affected by the light intensity, although it did slightly increase during shelf life in Sky rocket leaves. Glucoerucin was present in low levels in Sky rocket compared to those in Voyager; glucoiberverin showed the greatest decline during shelf life, particularly in Sky rocket, whereas glucoraphanin increased during the storage period in both species (**Figure 4**). Force et al. (*23*) have reported a decrease during shelf life of glucoerucin and glucoraphanin, whereas others (*6*) have reported glucoraphanin to be stable.

Glucosinolates are believed to confer their chemoprotective effect through the products of hydrolysis of these compounds by the enzyme myrosinase, which is present in the plant but spatially separated into different cellular compartments from glucosinolates. Two genes have been identified in the model cruciferous plant *Arabidopsis thaliana* that determine whether the hydrolysis products will be thiocyanates, which are of beneficial dietary importance to human consumers, or the less desirable nitriles. The presence of a functional *epithiospecifier* (*ESP*) gene leads to the formation of nitriles, but *ESP* is repressed by the presence of a functional *epithiospecifier modifier-1* (*ESM1*) gene (*24*), and isothiocyanates are then produced. Partial genomic sequences and ESTs for *ESP* and *ESM1* genes were cloned from Sky and Voyager rocket leaves (Sky *ESP*, FJ604594; Voyager *ESP*, FJ604595; Sky *ESM1*, FJ604597; Voyager *ESM1*, FJ604596) and compared to each other and to *Arabidopsis* orthologues to identify intron regions. Expression levels were determined using quantitative real time RT-PCR. There were no differences in expression profiles between cultivars of either gene, and *ESP* expression remained constant over shelf life. However, the expression of *ESM1* declined to approximately 30% of the level at harvest over the course of 14 days of shelf life (**Figure 4C**). This is consistent with the findings that glucoerucin and glucoraphanin declined during the shelf life of rocket (*23*) and our own finding that glucoerucin declined during the shelf life of wild rocket (but not salad rocket) and that glucosativin and glucoiberverin declined during the shelf life of both species. However, we have not yet established if the reduction in *ESM1* expression correlates with a change in the absolute levels or the proportions of nitrile or isothiocyanates compounds produced.

**The Protective Effect of Glucosinolate-Rich Extracts against $H_2O_2$-Induced DNA Damage Was Species Specific.** The effect of Sky and Voyager rocket leaf glucosinolate extracts on DNA damage and their potential to protect against $H_2O_2$-induced DNA damage were investigated in colon cancer HT-29 cells using the comet assay. Treatment with $H_2O_2$ for 5 min significantly induced DNA strand breaks with a tail DNA percentage of 35.42 ± 1.58% (**Figure 5A**). Neither Sky nor Voyager rocket leaf extracts rich in glucosinolates showed significant DNA damage inducing effect (data not shown). Pretreatment of HT-29 cells for 24 h with glucosinolate-rich extracts from Sky rocket, exposed to either high or low light, significantly inhibited subsequent $H_2O_2$-induced DNA strand breaks in HT-29 cells at all of the concentrations tested (**Figure 5A**), though not in a dose-dependent manner. In contrast, DNA damage induced by $H_2O_2$ was slightly inhibited by Voyager rocket glucosinolate extracts, but the decrease was not statistically significant. The protective effect of Sky rocket glucosinolate extracts cannot be attributed to their antioxidant capacities because leaf extracts rich in glucosinolates from Sky and Voyager rocket plants exposed to high light showed dramatically high antioxidant activities compared to that of their counterparts with low light exposure (**Figure 2**), and there was no observed correlation between antioxidant activity and protection against DNA damage. A study by Plumb et al. (*25*) also disputed the direct oxidant protection properties of GSLs as the direct antioxidant properties of extracts of cruciferous plants did not correlate with GSL content. Analysis of GSL content in this paper showed that rocket leaves exposed to high light produced much more GSLs than those grown in low light, yet the impact of leaf extracts from each growth condition showed no differential effect of antigenotoxicity in the colon cells, suggesting that GSLs might not be the major or only contributor to the protective effect observed for Sky rocket. However, we cannot rule out the possibility of synergism of certain GSLs in specific ratios, or beneficial interaction between different phytochemicals, as supported by a study on *Raphanus sativus* L., which demonstrated that plant extracts had a greater chemoprotective effect than their individual components (*26*). None of the extracts that were incubated with colon cells in our study caused cell death as determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown). As rocket leaves exposed to high light contained more phytochemicals such as glucosinolates and flavonoids, leaves from Sky and Voyager rocket cultivated under high light were chosen for further analysis.

Enzymatic hydrolysis of most glucosinolates is required for the formation of biologically active isothiocyanates (ITCs) and indoles (*1*). At neutral pH and a temperature >25 °C, the formation of ITCs is favored, and myrosinase is very stable under cell culture conditions (pH 7.4, 37 °C) (*17*). The addition of myrosinase to our in situ system mildly enhanced the inhibition of $H_2O_2$-induced DNA damage by Sky and Voyager rocket glucosinolate extracts (**Figure 5B**). The enhancement was more effective in the case of Sky rocket and statistically significant when the concentration of myrosinase was above 0.05 units mL$^{-1}$. Hydrolysis by myrosinase of glucosinolates in Voyager rocket extracts did not produce any statistically significant protection against $H_2O_2$ challenge. Bonnesen et al. (*27*) also showed that intact glucosinolates were more powerful phase I and II enzyme inducers than the in vitro myrosinase-degraded glucosinolates.

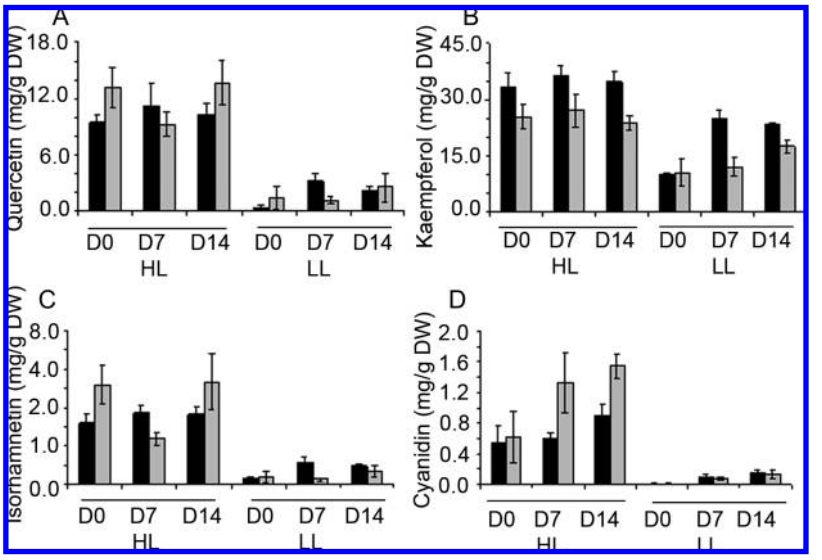


**Figure 3.** HPLC analysis of flavonoids in Sky (black bars) and Voyager (gray bars) grown in high light (HL) or low light (LL) at harvest (D0) and after seven (D7) and 14 days (D14) of storage at 4 °C. *N* = 3.

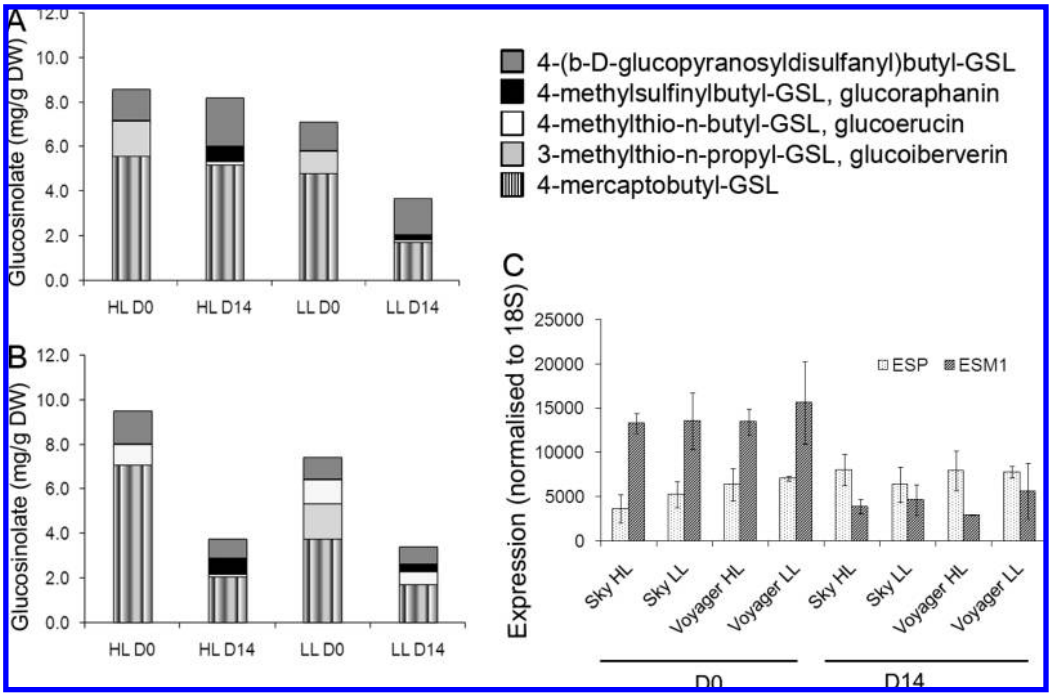


**Figure 4.** Glucosinolate profiles of (**A**) Sky and (**B**) Voyager rocket samples grown under high light (HL) or low light (LL) conditions at harvest (D0) or after 14 days of storage at 4 °C (D14). (**C**) Expression profiling of genes regulating glucosinolate hydrolysis. ESP, epithiospecifier; ESM1, epithiospecifier modifier 1 determined by quantitative RT-PCR. *N* = 3.

The HT-29 cell line used in the present study has a higher glutathione-*S*-transferase (GST) activity compared to primary colonocytes (*28*); thus, isothiocyanates formed through the hydrolysis of glucosinolates might be rapidly conjugated to glutathione via the action of GSTs and therefore not be available to offer protection against oxidative stress. This theory is supported by recent findings of Lamy et al. (*29*) who established that *E. sativa* extract, at a concentration showing antigenotoxic effect, induced a significant elevation of GST activity.

It was surprising that Sky rocket showed a protective effect on the colon cells regardless of the light intensity conditions for plant growth and the resulting differences in the quantity of phytochemicals. This indicates either that the protective effect of glucosinolates and their hydrolysis products might be counteracted by other phytochemicals present in Voyager rocket leaves or that a very small amount of glucosinolates is required to inititate cell defense mechanisms against oxidative stress, and therefore, increased concentrations do not have an additive effect. A recent ontogenic profiling of phytochemicals in several rocket species revealed that the major differences between the *Eruca* and *Diplotaxis* species were the presence of sinapine in *Diplotaxis* seeds and sprouts, which was absent from all *Eruca* species (*7*). Another report confirmed that a sinapic acid derivative was the only phenolic acid compound detected in the leaves of *Diplotaxis tenuifolia* (*4*). Sinapate ester compounds are considered antinutritional mainly due to the fact that they confer astringency and low digestibility of seed meal that is otherwise rich in proteins of a well-balanced composition for animal feeding and potential

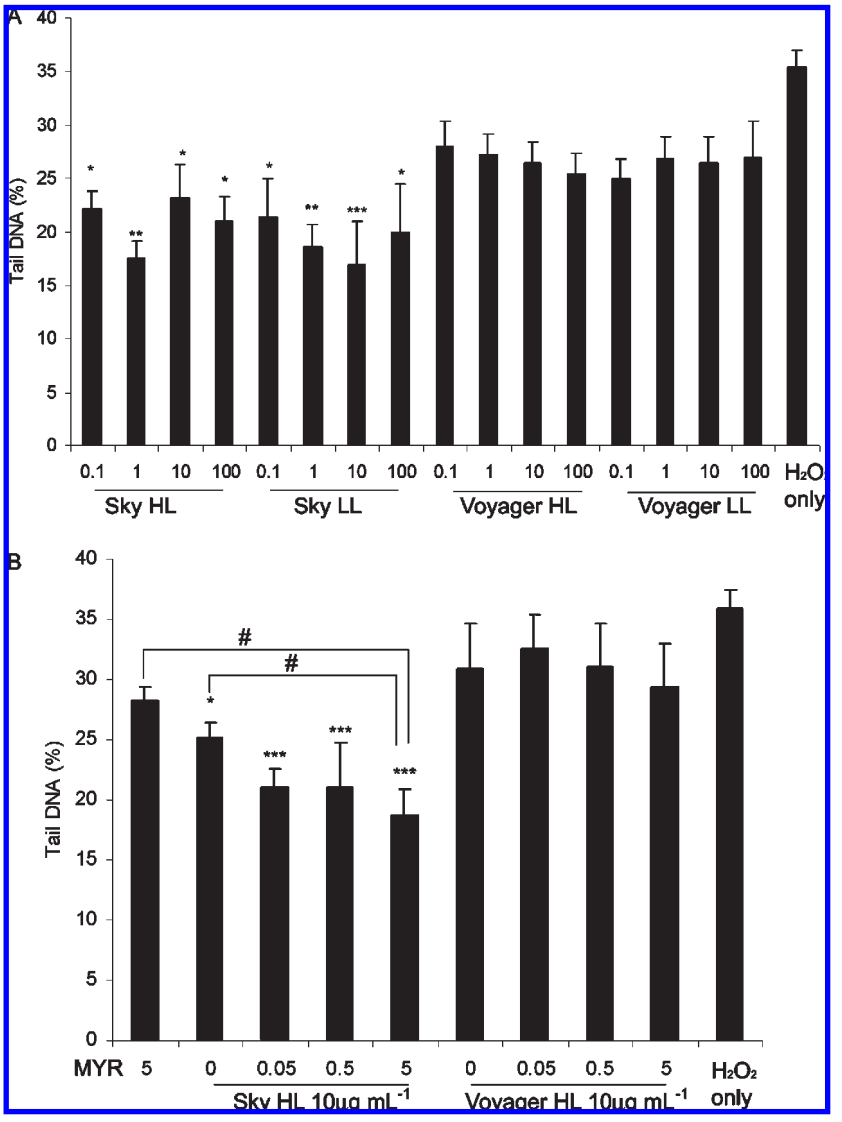


**Figure 5.** Protective effect of glucosinolate-rich extract from Sky Rocket leaves against $H_2O_2$-induced DNA damage: HT-29 cells were treated with glucosinolate extracts at different concentrations ($\mu$g DW mL$^{-1}$) as indicated (**A**) or glucosinolate extracts (10 $\mu$g DW mL$^{-1}$) with myrosinase at different concentrations (units mL$^{-1}$) (**B**) for 24 h before harvested and exposed to 75 $\mu$mol L$^{-1}$ $H_2O_2$ for 5 min. DNA damage was measured using the comet assay. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ vs $H_2O_2$ only. #, $P < 0.05$ as compared to myrosinase only (MYR 5 units mL$^{-1}$) and Sky rocket glucosinolate extract only (Sky HL). $N = 3$.

human food supplements. Further analysis of sinapine contents in mature leaves might shed light on the reason underlying the lack of antigenotoxicity in Voyager rocket.

**Flavonoid Aglycones from Rocket Leaves Showed Different Protection Patterns against $H_2O_2$ Damage.** HPLC data demonstrated that glucosinolate extracts from rocket leaves contained significant levels of polyglycosylated flavonoids (data not shown). As these glycosylated flavonoids cannot enter cells freely, acid hydrolyzed flavonoid extracts were tested for their genotoxicity and antigenotoxic effects using the comet assay. Flavonoid aglycone extracts from Sky rocket leaves exposed to high light at the concentration of 10 $\mu$g DW mL$^{-1}$ induced significant DNA strand break ($P < 0.001$ vs control), whereas the extracts from Voyager rocket showed no obvious DNA damage-inducing activity (**Figure 6A**). When the cells were pretreated for 24 h with Sky rocket flavonoid extracts, followed by $H_2O_2$ challenge, DNA damage induced by $H_2O_2$ was reduced (**Figure 6B**) in one of the concentrations studied. The genotoxicity of flavonoids such as kaempferol and quercetin has previously been recognized and noted, indicating that when these compounds are coadministered with mutagens, they become antigenotoxic (*30*). We also observed this in the case of Sky rocket, which probably was due to significantly higher contents of kaempferol inside its leaves compared to that in Voyager rocket leaves (**Figure 3B**). The overall antioxidant capacity in Sky and Rocket leaves was approximately equal (**Figure 2**); therefore, the antigenotoxic effect observed in flavonoid-rich extracts of Sky rocket is probably not mediated via antioxidant activity but is more likely to be due to a different mode of action of particular compounds within the mixture.

Although flavonoid aglycones showed a somewhat protective effect on $H_2O_2$-induced genetic damage, a significant effect was observed only at 1.0 mg DW mL$^{-1}$ for Sky rocket where the tail DNA was reduced from 35% (control) to 25% (flavonoid extract, **Figure 6**B), and they are therefore unlikely to contribute to the protection we observed for Sky rocket extracts rich in glucosinolates (tail DNA reduced to 17%, **Figure 5**A). One reason is that the majority of flavonoids were not present as aglycones, an observation confirmed by Bennett et al. (*7*). Bennett et al. also revealed using LC/MS analyses the presence of the N-heterocycles in *Eruca* species that were absent from all *Diplotaxis* seeds and seedlings. Cataldi et al. (*8*) used optimized reversed-phase

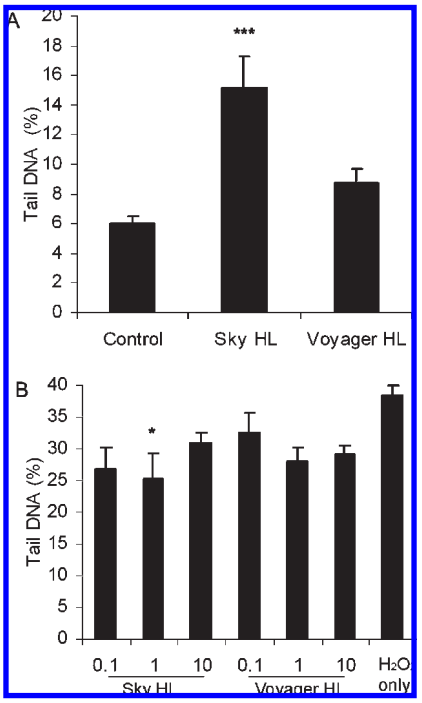


**Figure 6.** Effect of flavonoid aglycone extracts from rocket leaves on DNA damage. (**A**) HT-29 cells were treated with flavonoid rocket extracts at 10 $\mu$g DW mL$^{-1}$ for 24 h before harvesting and analysis of DNA damage using the comet assay. ***, $P < 0.001$ vs control. (**B**) HT-29 cells were treated with flavonoid rocket extract at the indicated concentrations ($\mu$g DW mL$^{-1}$) for 24 h before harvested and exposed to 75 $\mu$M $H_2O_2$ for 5 min. DNA damage was measured using the comet assay. *, $P < 0.05$ vs $H_2O_2$ only. $N = 3$.

liquid chromatography (RP-LC) with electrospray ionization (ESI) ion trap mass spectrometry (ITMS) to analyze naturally occurring glucosinolates in crude extracts of *Eruca sativa* L. and revealed three indole glucosinolates (i.e., three N-heterocyclic compounds), although we did not identify any indolyl glucosinolates in the tested rocket extracts. The function of N-heterocycles identified in Bennett's study and their possible role in the protection against $H_2O_2$-induced DNA damage warrants further investigation.

In conclusion, our study provides evidence that both plant growth conditions and postharvest storage conditions have an impact on the range and quantity of phytonutrients accumulated in leaves that are important in human health. We have established that there are substantial differences between the two genera of leafy cruciferous vegetables commonly known as rocket and made the important finding that the salad rocket (*Eruca sativa*) may have a greater potential for positive impact on human health than the wild rocket. However, further work is required to establish whether the differences observed in glucosinolate profiles do correlate with variation in the composition or quantity of their hydrolysis products. Our findings that flavonoid profiles vary with genotype and environment provide some interesting directions for future plant breeding programs and indicate that controlled environmental stress may have beneficial effects on the accumulation of valuable phytonutrients in plants that are consumed in the human diet.

## ABBREVIATIONS USED

GSL, glucosinolate; $H_2O_2$, hydrogen peroxide; ITC, isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FW, fresh weight; DW, dry weight.

**Supporting Information Available:** Tabulated data shown in Figures 2−4. This material is available free of charge via the Internet at http://pubs.acs.org.

## LITERATURE CITED

(1) Higdon, J. V.; Delage, B.; Williams, D. E.; Dashwood, R. H. Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. *Pharmacol. Res.* **2007**, *55*, 224–236.
(2) Aggarwal, B. B.; Shishodia, S. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem. Pharmacol.* **2006**, *71*, 1397–1421.
(3) Fahey, J. W.; Zalcmann, A. T.; Talalay, P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* **2001**, *56*, 5–51.
(4) Martínez-Sánchez, A.; Marín, A.; Llorach, R.; Ferreres, F.; Gil, M. I. Controlled atmosphere preserves quality and phytonutrients in wild rocket (*Diplotaxis tenuifolia*). *Postharvest Biol. Technol.* **2006**, *40*, 26–33.
(5) Simmonds, M. S. Flavonoid-insect interactions: recent advances in our knowledge. *Phytochemistry* **2003**, *64*, 21–30.
(6) Winkler, S.; Faraghera, J.; Franza, P.; Imsica, M.; Jones, R. Glucoraphanin and flavonoid levels remain stable during simulated transport and marketing of broccoli (*Brassica oleracea* var. italica) heads. *Postharvest Biol. Technol.* **2007**, *43*, 89–94.
(7) Bennett, R. N.; Rosa, E. A.; Mellon, F. A.; Kroon, P. A. Ontogenic profiling of glucosinolates, flavonoids, and other secondary metabolites in *Eruca sativa* (salad rocket), *Diplotaxis erucoides* (wall rocket), *Diplotaxis tenuifolia* (wild rocket), and *Bunias orientalis* (Turkish rocket). *J. Agric. Food Chem.* **2006**, *54*, 4005–4015.
(8) Cataldi, T. R.; Rubino, A.; Lelario, F.; Bufo, S. A. Naturally occurring glucosinolates in plant extracts of rocket salad (*Eruca sativa* L.) identified by liquid chromatography coupled with negative ion electrospray ionization and quadrupole ion-trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2374–2388.
(9) Perocco, P.; Iori, R.; Barillari, J.; Broccoli, M.; Sapone, A.; Affatato, A.; Paolini, M. *In vitro* induction of benzo(a)pyrene cell-transforming activity by the glucosinolate gluconasturtiin found in cruciferous vegetables. *Cancer Lett.* **2002**, *184*, 65–71.
(10) Hintze, K. J.; Keck, A. S.; Finley, J. W.; Jeffery, E. H. Induction of hepatic thioredoxin reductase activity by sulforaphane, both in Hepa1c1c7 cells and in male Fisher 344 rats. *J. Nutr. Biochem.* **2003**, *14*, 173–179.
(11) Martínez-Sanchez, A.; Llorach, R.; Gil, M. I.; Ferreres, F. Identification of new flavonoid glycosides and flavonoid profiles to characterize rocket leafy salads (*Eruca vesicaria* and *Diplotaxis tenuifolia*). *J. Agric. Food Chem.* **2007**, *55*, 1356–1363.
(12) Arts, I. C.; Hollman, P. C. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **2005**, *81*, 317S–325S.
(13) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231–1237.
(14) Wagstaff, C.; Yang, T. J. W.; Stead, A. D.; Buchanan-Wollaston, V.; Roberts, J. A. A molecular and structural characterisation of senescing Arabidopsis siliques and comparison of transcriptional profiles with senescing petals and leaves. *Plant J.* **2009**, *57*, 690–705.
(15) Wagstaff, C.; Leverentz, M.; Griffiths, G.; Thomas, B.; Chanasut, U.; Stead, A.; Rogers, H. Cysteine protease gene expression and proteolytic activity during senescence of *Alstroemeria* petals. *J. Exp. Bot.* **2002**, *53*, 233–240.
(16) Gill, C. I.; Boyd, A.; McDermott, E.; McCann, M.; Servili, M.; Selvaggini, R.; Taticchi, A.; Esposto, S.; Montedoro, G.; McGlynn, H.; Rowland, I. Potential anti-cancer effects of virgin olive oil phenols on colorectal carcinogenesis models *in vitro*. *Int. J. Cancer* **2005**, *117*, 1–7.
(17) Leoni, O.; Iori, R.; Palmieri, S.; Esposito, E.; Menegatti, E.; Cortesi, R.; Nastruzzi, C. Myrosinase-generated isothiocyanate from glucosinolates: isolation, characterization and in vitro antiproliferative studies. *Bioorg. Med. Chem.* **1997**, *5*, 1799–1806.
(18) Gould, K. S. Nature's Swiss Army Knife: The diverse protective roles of anthocyanins in leaves. *J. Biomed. Biotechnol.* **2004**, *2004*, 314–320.

(19) Lichtenthaler, H. K.; Buschmann, C.; Döll, M.; Fietz, H.-J.; Bach, T.; Kozel, U.; Meier, D.; Rahmsdorf, U. Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. *Photosynth. Res.* **1981**, *2*, 115–141.

(20) Bartley, G. E.; Scolnik, P. A. Plant carotenoids: pigments for photoprotection, visual attraction, and human health. *Plant Cell* **1995**, *7*, 1027–1038.

(21) Bergquist, S.Å.M.; Gertsson, U. E.; Olsson, M. E. Influence of growth stage and postharvest storage on ascorbic acid and carotenoid content and visual quality of baby spinach. *J Sci. Food Agric.* **2006**, *86*, 346–355.

(22) Viña, S. Z.; Chaves, A. R. Antioxidant responses in minimally processed celery during refrigerated storage. *Food Chem.* **2006**, *94*, 68–74.

(23) Force, L. E.; O'Hare, T. J.; Wong, L. S.; Irving, D. E. Impact of cold storage on glucosinolate levels in seed-sprouts of broccoli, rocket, white radish and kohl-rabi. *Postharvest Biol. Technol.* **2007**, *44*, 175–178.

(24) Zhang, Z.; Ober, J. A.; Kliebenstein, D. J. The gene controlling the quantitative trait locus EPITHIOSPECIFIER MODIFIER1 alters glucosinolate hydrolysis and insect resistance in *Arabidopsis*. *Plant Cell* **2006**, *18*, 1524–1536.

(25) Plumb, G. W.; Lambert, N.; Chambers, S. J.; Wanigatunga, S.; Heaney, R. K.; Plumb, J. A.; Aruoma, O. I.; Halliwell, B.; Miller, N. J.; Williamson, G. Are whole extracts and purified glucosinolates from cruciferous vegetables antioxidants? *Free Radic. Res.* **1996**, *25*, 75–86.

(26) Barillari, J.; Iori, R.; Papi, A.; Orlandi, M.; Bartolini, G.; Gabbanini, S.; Pedulli, G. F.; Valgimigli, L. Kaiware Daikon (*Raphanus sativus* L.) extract: A naturally multipotent chemopreventive agent. *J. Agric. Food Chem.* **2008**, *56*, 7823–7830.

(27) Bonnesen, C.; Stephensen, P. U.; Andersen, O.; Sorensen, H.; Vang, O. Modulation of cytochrome P-450 and glutathione S-transferase isoform expression in vivo by intact and degraded indolyl glucosinolates. *Nutr. Cancer* **1999**, *33*, 178–187.

(28) Ebert, M. N.; Klinder, A.; Peters, W. H.; Schaferhenrich, A.; Sendt, W.; Scheele, J.; Pool-Zobel, B. L. Expression of glutathione S-transferases (GSTs) in human colon cells and inducibility of GSTM2 by butyrate. *Carcinogenesis* **2003**, *24*, 1637–1644.

(29) Lamy, E.; Schroder, J.; Paulus, S.; Brenk, P.; Stahl, T.; Mersch-Sundermann, V. Antigenotoxic properties of *Eruca sativa* (rocket plant), erucin and erysolin in human hepatoma (HepG2) cells towards benzo(a)pyrene and their mode of action. *Food Chem. Toxicol.* **2008**, *46*, 2415–2421.

(30) Anderson, D.; Dobrzynska, M. M.; Basaran, N.; Basaran, A.; Yu, T. W. Flavonoids modulate comet assay responses to food mutagens in human lymphocytes and sperm. *Mutat. Res.* **1998**, *402*, 269–277.

**Received January 26, 2009. Revised manuscript received April 8, 2009. Accepted May 11, 2009.**