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# *Brassica oleracea* L. Var. *costata* DC and *Pieris brassicae* L. Aqueous Extracts Reduce Methyl Methanesulfonate-Induced DNA Damage in V79 Hamster Lung Fibroblasts

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**ABSTRACT:** *Brassica oleracea* L. var. *costata* DC leaves and *Pieris brassicae* L. larvae aqueous extracts were assayed for their potential to prevent/induce DNA damage. None of them was mutagenic at the tested concentrations in the Ames test reversion assay using *Salmonella* His$^+$ TA98 strains, with and without metabolic activation. In the hypoxanthine-guanine phosphoribosyltransferase mutation assay using mammalian V79 fibroblast cell line, extracts at 500 $\mu$g/mL neither induced mutations nor protected against the mutagenicity caused by methyl methanesulfonate (MMS). In the comet assay, none of the extracts revealed to be genotoxic by itself, and both afforded protection, more pronounced for larvae extracts, against MMS-induced genotoxicity. As genotoxic/antigenotoxic effects of *Brassica* vegetables are commonly attributed to isothiocyanates, the extracts were screened for these compounds by headspace−solid-phase microextraction/gas chromatography−mass spectrometry. No sulfur compound was detected. These findings demonstrate that both extracts could be useful against damage caused by genotoxic compounds, the larvae extract being the most promising.

**KEYWORDS:** *Pieris brassicae larvae, Brassica oleracea var. costata, genoprotection*

## INTRODUCTION

Organisms are exposed to a multitude of compounds that are able to cause DNA damage, directly or after biotransformation. Mutation occurs if the genetic material changes in a permanent transmissible way.[1,2] Genotoxicity refers to potentially harmful effects on the genetic material of cells, which are not necessarily associated with mutagenicity. These changes may involve a single gene or gene segment, a block of genes, or whole chromosomes.

Fruits and vegetables, including the ones used in human nutrition, are considered to exert antimutagenic effects against a variety of mutagenic compounds. On the other hand, diet-related mutagenesis plays an etiologic role in chronic diseases, as food plants can be mutagenic by themselves.[2] Ames suggested that natural chemicals, present in the human diet as complex mixtures, may be a more important source of human mutation than environmental or occupational exposures.[3]

Epidemiological studies provide evidence that cruciferous vegetables protect humans against cancer.[4] Furthermore, results from animal experiments demonstrate that they reduce chemically induced tumor formation. These properties have been attributed to alterations in the metabolism of carcinogens by breakdown products of glucosinolates.[5] Other secondary compounds ubiquitously distributed in plants can also contribute to the general effect. Phenolic compounds comprise many examples of antimutagens acting by various mechanisms, including the impairment of metabolic activation of various pro-carcinogens. Both mutagenic and antimutagenic properties have been ascribed to several members of this group.[2]

Volatile compounds deriving from several biosynthetic pathways can have important bioactivities. Nonconjugated plant volatiles are lipophilic molecules with high vapor pressure, allowing them to cross membranes freely and evaporate into the atmosphere, where there are no barriers to diffusion.[6] Thus, they can be easily absorbed by cells and exert either protective or deleterious effects.

Phenolics and low molecular weight compounds have been described in the leaves of *Brassica oleracea* L. var. *costata* DC.[7,8] However, apart from sulfur compounds, information on their

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Table 1. Volatile Composition of *P. brassicae* Larvae and *B. oleracea* Var. *costata* Leaves Aqueous Extracts (μg/kg)

| | | | *P. brassicae* | | *B. oleracea* var. *costata* | |
|---|---|---|---|---|---|---|
| compd | quantification ions (*m*/*z*) | response factors | mean | SD | mean | SD |
| octanal | 43/56/69/84 | $7.8 \times 10^6$ | 32.377[c] | 3.223 | 85.487 | 4.996 |
| *trans*-geranylacetone | 69/107/151/194 | $6.6 \times 10^6$ | 21.630[a] | 2.241 | 11.612 | 1.091 |
| eugenol | 77/131/164 | $4.7 \times 10^5$ | 37.185[c] | 1.891 | 232.546 | 12.140 |
| β-cyclocitral | 109/137/152 | $5.3 \times 10^7$ | 4.171 | 0.417 | 1.341 | 0.103 |
| (−)-menthol | 71/81/95/123/155 | $1.5 \times 10^7$ | 13.358[b] | 0.016 | 30.094 | 0.408 |
| β-ionone | 43/91/135/177 | $1.9 \times 10^6$ | 58.098[c] | 4.624 | 85.569 | 2.227 |

[a] $P \leq 0.05$. [b] $P \leq 0.001$. [c] $P \leq 0.0001$, as compared to *B. oleracea* var. *costata* for each compound.

contribution to mutagenicity induction or protection by *Brassica* vegetables is still scarce.

Larvae of *Pieris brassicae* L. (Lepidoptera: Pieridae) are specialists on crucifers, feeding on a variety of Brassicaceae species, including *B. oleracea* var. *costata*. The larvae metabolize and accumulate secondary metabolites from their host plant,[9] thus deserving to be screened in terms of their biological effects. In this way, the undesirable effects of the plague in the crops yields could be counterbalanced by the use of the plague itself for obtaining bioactive compounds with potential applications as dietary supplements or in food and pharmaceutical industries.

Previously, the aqueous extracts of larvae and host plant were found to act as scavengers of several reactive oxygen species (superoxide and hydroxyl radicals), with the larvae extract being the most effective. In addition, the larvae extract exhibited a strong inhibitory effect on xanthine oxidase that was not observed for *B. oleracea* var. *costata* leaves.[10] The aqueous extract of *P. brassicae* larvae fed with another *B. oleracea* variety (var. *acephala*) also was revealed to be more effective in scavenging nitric oxide radical than the host plant.[11] Because reactive oxidative species can cause a range of DNA lesions, the ability of the extracts to scavenge them is expected to afford some DNA protection, for which other mechanisms can also contribute.[2]

In this work, we intended to evaluate the potential of aqueous extracts of *B. oleracea* var. *costata* leaves and *P. brassicae* larvae to induce DNA damage, using short-term in vitro assays involving bacteria (*Salmonella typhimurium* TA98) and mammalian cells (V79 Chinese hamster lung fibroblast cell line). Furthermore, the potential of the extracts to protect against the genotoxin methyl methanesulfonate (MMS) was evaluated. V79 cells lack CYP activity, so MMS was chosen because it is a direct alkylating agent that does not require biotransformation to produce DNA damage.[12] This DNA alkylating agent has been used for many years as a DNA damaging agent to induce mutagenesis and in recombination experiments. MMS modifies both guanine (to 7-methylguanine) and adenine (to 3-methlyladenine) to cause base mispairing and replication blocks, respectively.[13]

Mutagenicity/antimutagenicity in mammalian cells was assessed using the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene mutation assay, and the comet assay (single-cell gel electrophoresis) was used to evaluate the genotoxic/antigenotoxic effects at the cell level, since not all DNA lesions result in a mutational signature.

## MATERIALS AND METHODS

**Standards and Reagents.** Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and other biochemicals were obtained from Invitrogen (Gibco, United States). S9 mix was from Trinova Biochem (Giessen, Germany). Chemicals and solvents were of analytical grade and purchased from Merck (Darmstadt, Germany), Sigma, (St. Louis, MO), SAFC (Steinheim, Germany), and Fluka (Buchs, Switzerland). Standards of octanal, *trans*-geranylacetone, β-cyclocitral, and eugenol were from Sigma-Aldrich (St. Louis, MO); β-ionone was obtained from SAFC, and menthol was from Fluka. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

**Samples.** *B. oleracea* var. *costata* leaves and *P. brassicae* larvae developed until the fourth instar were grown in greenhouses and collected in November 2008 as previously described.[7] Larvae subjected to 1 h of starvation and its host plant (collected after *P. brassicae* larvae predation) were freeze-dried, powdered, and kept in a desiccator in the dark until analysis. Voucher specimens were deposited at the laboratory of Pharmacognosy from Faculty of Pharmacy of Porto University.

**Extracts Preparation.** Aqueous extracts were prepared by decoction of ca. 1 g sample in 800 mL of water for 30 min, according to a previously described method.[7] The obtained extract was sequentially cooled, filtered, lyophilized, and kept in a desiccator in the dark until analysis.

**Headspace Solid-Phase Microextraction (HS-SPME).** Approximately 75 mg of lyophilized extract was dissolved in 5 mL of water and submitted to SPME as described before.[9] Samples were stirred (150 rpm) at 40 °C for 5 min. The fiber (divinylbenzene/PDMS coating, 50/30 μm) was then exposed to the headspace for 20 min. Afterward, the fiber was pulled into the needle sheath and inserted into the injection port of the GC system for thermal desorption during 1 min. Standards were analyzed under the same conditions. Samples and standards were assayed in triplicate.

**Gas Chromatography–Mass Spectrometry (GC-MS) Analysis.** Analysis was performed using a Varian CP-3800 gas chromatograph (United States) equipped with a Varian Saturn 4000 mass selective detector and a Saturn GC/MS workstation software version 6.8. The column was VF-5 ms (30 m × 0.25 mm × 0.25 μm) from Varian. The injector port was heated to 220 °C, and injections were performed in splitless mode. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. The oven temperature was set at 40 °C for 1 min, increasing 2 °C/min to 220 °C, and held for 30 min. All mass spectra were acquired in the electron impact (EI) mode in the range of 40–350 *m*/*z*, with scan speed of 6 scan/s. During the first minute, ionization was maintained off. The ion trap detector was set as follows: the transfer line, manifold, and trap temperatures were, respectively, 280, 50, and 180 °C. The emission current was 50 μA, and the electron multiplier was set in relative mode to autotune procedure. The maximum ionization time was 25000 μs, with an ionization storage level of 35 *m*/*z*. Identification of components was made on the basis of their retention indices relative to $C_8$–$C_{20}$ *n*-alkanes indices and mass spectra, which were compared with those of NIST 05 MS Library Database (Match and R.Match >80%), pure standards analyzed under the same conditions and NIST Chemistry WebBook. Peak areas were determined by reconstructed full-scan chromatograms using for each compound some specific ions (Table 1). Quantification was achieved by the external standard method. Three determinations were performed.

Table 2. Viability (%) of V79 Cells Exposed to *P. brassicae* Larvae and *B. oleracea* Var. *costata* Leaves Aqueous Extracts[a]

| | *B. oleracea* var. *costata* | | | | *P. brassicae* | | | |
|---|---|---|---|---|---|---|---|---|
| | MTT assay | | LDH assay | | MTT assay | | LDH assay | |
| extract ($\mu$g/mL) | mean | SE | mean | SE | mean | SE | mean | SE |
| 4 | 99.22 | 2.85 | 91.47 | 6.14 | 107.42 | 3.53 | 96.66 | 0.96 |
| 20 | 99.03 | 3.94 | 92.10 | 5.00 | 104.60 | 6.20 | 97.87 | 2.60 |
| 100 | 100.19 | 3.83 | 89.57 | 2.41 | 104.26 | 5.76 | 93.82 | 5.81 |
| 500 | 97.64 | 2.27 | 93.43 | 4.86 | 106.32 | 7.84 | 96.36 | 2.20 |

[a]Differences between the two samples, for the same assay and same concentration, were not significant. For the same sample, differences between the two assays were not significant. In each assay, differences between extract concentrations were not significant.

**Cell Culture and Treatments.** V79 cells (Chinese hamster lung fibroblasts) were cultured in DMEM, containing 10% heat-inactivated FBS, penicillin/streptomycin (100 U/mL/100 $\mu$g/mL), and 1% nonessential amino acids, in a humidified incubator at 37 °C and 5% $CO_2$. Cells were seeded at a density of $1 \times 10^4$ cells/cm$^2$. After confluence, cells were exposed to extracts in concentrations ranging from 4 to 500 $\mu$g/mL for 24 h. Standard volatile compounds dissolved in DMSO were tested in the concentration range of 1−1000 $\mu$g/mL. The final concentration of DMSO (0.1% v/v) did not affect cellular viability.

**V79 Cells Viability Assays.** *(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Reduction Assay.* Cell viability was assessed by the reduction of MTT to formazan, as described before.[14] Briefly, after exposure, the medium was removed, and the cells were incubated for 30 min at 37 °C in medium containing 0.5 mg/mL MTT. Then, medium containing MTT was removed, formazan crystals were solubilized with DMSO, and the resulting purple solution was measured spectrophotometrically at 570 nm. Data are presented as the percentage of MTT reduction of treated cells relative to the untreated ones. Three independent assays were conducted, each one of them in triplicate.

*Lactate Dehydrogenase (LDH) Leakage Assay.* Briefly, the release of the cytosolic enzyme LDH into the culture medium was evaluated as follows: after 24 h of exposure, an aliquot of the culture medium was taken and mixed with a NADH and pyruvate buffered solution. The LDH activity was measured spectrophotometrically by following the conversion of NADH to $NAD^+$ at 340 nm.[15] Results are expressed in percentage of treated cells relative to the untreated ones of three independent experiments, performed in triplicate.

**In Vitro Mutagenicity Assays.** *Ames Test.* The assay was performed with the TA98 tester strain of *S. typhimurium*, according to the method described by Ames.[3] Lyophilized extracts dissolved in water were tested from 10 to 1000 $\mu$g/plate. Benzo[*a*]pyrene (B[a]P) at 5 ng/plate and quercetin at 10 $\mu$g/plate, dissolved in dimethyl sulfoxide (DMSO), were used as positive controls in the presence and absence of metabolic activation (S9 mix), respectively. Approximately 880 and 288 revertents were obtained as positive control for B[a]P and quercetin, respectively. Negative controls were also performed, according to the solvent used to prepare extracts and standards. The assays were carried out in triplicate.

*HPRT Assay.* HPRT gene mutation assay was performed according to a previously described method, with some modifications.[16] Briefly, V79 cells pretreated with the aqueous extracts for 24 h were exposed to vehicle or 250 $\mu$M MMS, for 4 h. Then, cells were detached, and $2 \times 10^6$ cells were seeded. To ensure the expression of mutants, cells were maintained in culture for 7 days, being subcultured as soon as they reached confluence. After this period, the cultures were split into parallel subcultures, being $2 \times 10^5$ cells replated into Petri dishes with selective medium (10 $\mu$g/mL 6-thioguanine) and 250 cells in nonselective medium. Seven days later the 6-thioguanine-resistant colonies and the colonies grown in nonselective medium were fixed with ethanol:acetone (50:50), Giemsa stained, and counted. Three independent experiments were performed in triplicate for each extract, and the mean of mutants/$10^6$ cells were calculated.

**In Vitro Genotoxicity Assay.** *Comet Assay.* The genotoxic effects of the aqueous extracts in V79 cells were evaluated by the alkaline version of comet assay. Antigenotoxic effects against MMS alkylating agent were also assessed. The assays were performed as previously described by Singh and collaborators,[17] with the modifications introduced by Costa and co-workers.[18] Briefly, V79 cells were exposed to the extracts for 24 h, washed, and detached. For antigenotoxic assays, cells pretreated with the aqueous extracts were further exposed to 250 $\mu$M MMS for 2 h. After trypsinization, cells were collected, and viability, assessed by the trypan blue exclusion method, was always higher than 95%. About $5 \times 10^4$ cells were diluted in 0.6% low melting point agarose in PBS (pH 7.4) and dropped onto a frosted slide, precoated with a 1% normal melting point agarose layer. Slides were then allowed to solidify. Afterward, slides were immersed in lysing solution and placed on a horizontal electrophoresis tank, filled with alkaline electrophoresis solution (pH 13). Slides were left for 20 min in the dark. Electrophoresis was carried out for 20 min at 30 V and 300 mA (1 V/cm). The slides were then washed with neutralizing solution (pH 7.5) and stained with ethidium bromide solution (20 $\mu$g/mL). Two slides were prepared for each sample, and a blind scorer examined 50 randomly selected cells from each slide using a magnification of 400×. Image capture and analysis were performed with Comet Assay IV software (Perceptive Instruments). Comet tail intensity was the DNA damage parameter evaluated.

**Statistical Analysis.** Comparisons were performed by one-way and two-way analysis of variance (ANOVA), with the Bonferroni posthoc test, using GraphPad Prism 5 software.

## RESULTS

**Volatiles Characterization.** The aqueous extracts of *B. oleracea* var. *costata* leaves and *P. brassicae* larvae contained some low molecular weight compounds, as screened by HS-SPME/GC-MS technique. One aldehyde resulting from the degradation of fatty acids (octanal), two norisoprenoids ($\beta$-cyclocitral and $\beta$-ionone), two terpenoids [*trans*-geranylacetone and (−)-menthol], and one phenylpropanoid (eugenol) were determined (Table 1). Significant differences were observed between the content of each compound in each aqueous extract, except for $\beta$-cyclocitral. It can be seen that eugenol is the compound showing the most different content (6-fold higher in *B. oleracea* var. *costata* leaves extract).

*P. brassicae* larvae presented higher amounts of $\beta$-cyclocitral and *trans*-geranylacetone than *B. oleracea* host plant. $\beta$-Cyclocitral is a norisoprenoid, a class of compounds that results from carotenoids breakdown.[19] The accumulation of carotenoids by insects is well-known, mainly due to their antioxidant and pigmentation properties.[20] However, when it comes to norisoprenoids, little information is available. These results seem to provide evidence of the sequestration and/or bioconcentration of these compounds by *P. brassicae* larvae. Another possibility is that the insect itself is able to break the carotenoids present in kale into the detected norisoprenoids. In fact, this ability has been described in insects before, namely, in *Drosophila melanogaster*.[20]

*trans*-Geranylacetone was previously described by our group as an important terpenoid involved in defense of plant attacked by *P. brassicae*.[21] As observed for several compounds, this insect showed the capacity to take benefit from the diet, sequestering and accumulating several compounds, namely, terpenoids, for its own defense.[9] So, the accumulation of *trans*-geranylacetone by *P. brassicae* can explain the higher amounts of this compound in its extract.

Using the HS technique, no sulfur compound derived from glucosinolates was detected in the extracts. In fact, we have searched specifically for allyl isothiocyanate, because it was before identified in leaves of *B. oleracea* varieties[8,21,22] and in *P. brassicae* larvae[9,21] and was referred as being the responsible for the mutagenicity of crude juices of *Brassica* vegetables,[23] but it was not found in our extract, which was confirmed when the pure standard was analyzed under the same conditions.

**V79 Cells Viability Assays.** The extracts were not cytotoxic to V79 cells at the concentrations range tested (maximum 500 $\mu$g/mL), as verified by MTT and LDH assays (Table 2). No significant differences were observed between the two assays and among concentrations in the same assay. The posterior exposure to 250 $\mu$M MMS did not affect cell viability (data not shown).

The volatile compounds determined in the extracts were cytotoxic at 1 mg/mL, in both MTT and LDH assays ($P < 0.001$), with eugenol revealing to be already toxic at 100 $\mu$g/mL in the MTT assay ($P < 0.001$) (Figure 1).

**In Vitro Mutagenicity Assays.** *Ames Test.* The aqueous extracts did not induce a significant increase in mutation rates, neither in the absence nor in the presence of the external metabolizing enzyme system (S9 fraction from rat liver) (Figure 2). The spontaneous mutation rates were in the range of historical controls, and the respective positive controls indicated successful performance of the assay.

Low concentrations of the low molecular weight compounds present in the aqueous extracts (eugenol, <1 $\mu$g/plate; octanal, <10 $\mu$g/plate; $\beta$-cyclocitral, <25 $\mu$g/plate; and $\beta$-ionone, <50 $\mu$g/plate) exerted antimicrobial activity, as ascertained by the lower density of the bacterial background lawn (data not shown). So, it was not possible to evaluate their mutagenicity by using the Ames test.

*HPRT Assay.* The mutation frequency in V79 cells exposed to the extracts did not rise above the spontaneous level (Table 3). The long-time survival of cells previously exposed to the highest concentration of the aqueous extracts was also evaluated. In accordance with the MTT and LDH results, the colony-forming ability of the cells was similar to that of the control (data not shown).

MMS at 250 $\mu$M significantly induced mutagenicity to V79 cells ($P \leq 0.001$). The extracts (500 $\mu$g/mL) neither protected nor aggravated the mutations caused by MMS (Table 3).

**In Vitro Genotoxicity Assay.** *Comet Assay.* None of the aqueous extracts was genotoxic at the tested concentrations (Figure 3). Furthermore, the larvae aqueous extract protected V79 cells against the genotoxicity induced by MMS, and a tendency to decrease the deleterious effect of MMS was also observed with the *B. oleracea* var. *costata* one (Figure 3).

## DISCUSSION

The anticarcinogenic potential of Brassicaceae vegetables is generally attributed to their content in glucosinolates derivatives. As already referred, although such compounds were previously identified in leaves of *B. oleracea* varieties[8,21,22]

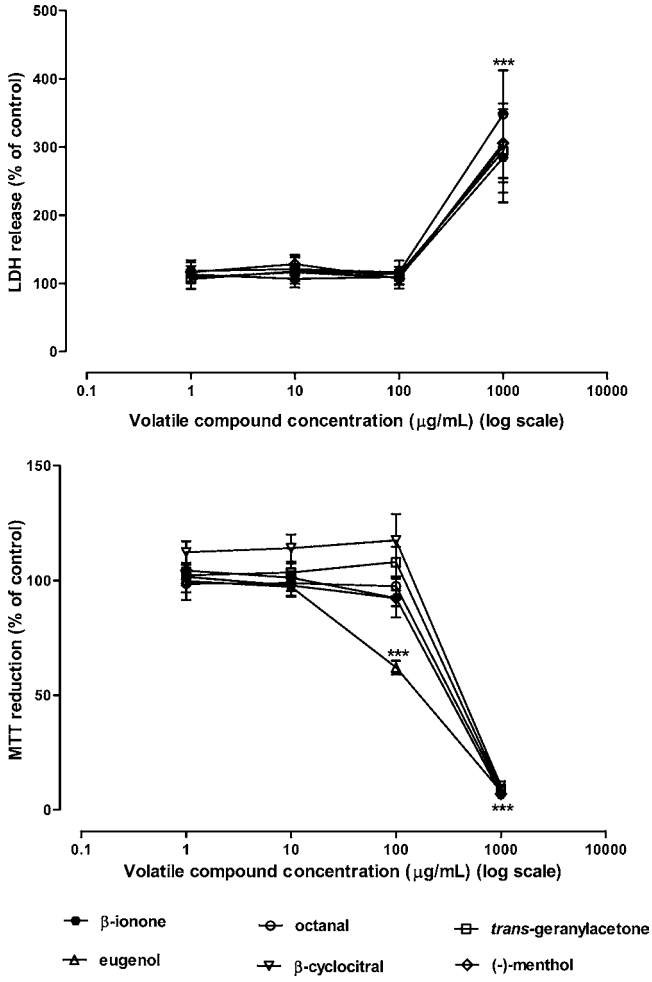


**Figure 1.** Effect of volatile compounds on MTT reduction and LDH release by V79 cells. Results correspond to the percentage of control (mean ± SEM). ***Mean values were significantly different as compared with control ($P < 0.001$).

and in *P. brassicae* larvae,[9,21] they were not detected in the aqueous extracts studied herein. This could be due to the loss of glucosinolates and breakdown products by volatilization during extracts' preparation, especially because the tissues were previously disrupted and the extracts were prepared with prolonged heating.[24] Aqueous extracts were used in this work because most vegetables are consumed after cooking; therefore, it is important to evaluate the mutagenic potential of extracts that mimic the usual procedure. Although larvae are not consumed after cooking, its extract was prepared in the same way to turn the comparison with that of *B. oleracea* var. *costata* more accurate and considering that it could be a potential source of bioactive compounds.

The cytotoxicity of the extracts should be determined before evaluating their mutagenicity, by using an appropriate indicator of cell integrity and growth, in a preliminary range-finding experiment. Because DNA damage is associated with cell death, it is critical that the highest dose tested does not induce excessive cytotoxicity. For concentrations between 0 and 500 $\mu$g/mL, no cytotoxicity was observed, and this range was chosen for the subsequent assays with V79 cells (Table 2).

The HS-SPME/GC-MS analysis of the extracts showed that they contain some low molecular weight compounds (Table 1), some of them already reported in *B. oleracea* var. *costata* leaves

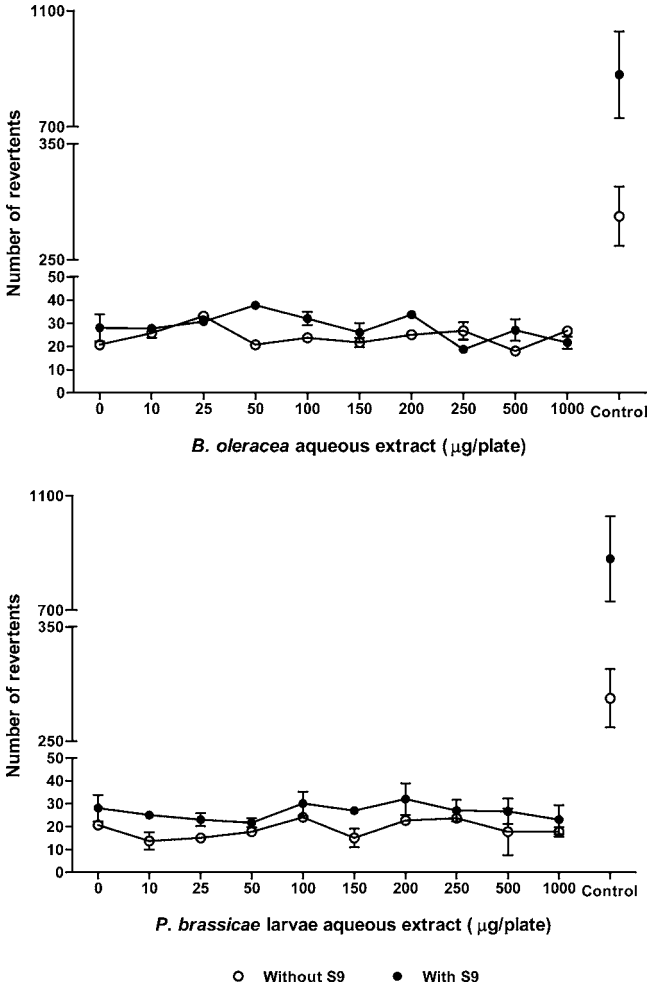


**Figure 2.** Ames test with *S. typhimurium* TA98 strain exposed to the aqueous extracts, performed with and without metabolic activation. Results are expressed as means ± SEMs.

**Table 3. Mutation Frequency/1 × $10^6$ Cells in the HPRT Assay in V79 Cells Exposed to *P. brassicae* Larvae and *B. oleracea* Var. *costata* Leaves Aqueous Extracts (500 μg/mL) with or without MMS (250 μM)**

| | without MMS | | with MMS | |
|---|---|---|---|---|
| | mean | SE | mean | SE |
| control | 8.89 | 2.74 | 50.00[a] | 7.99 |
| *B. oleracea* var. *costata* | 3.89 | 2.74 | 72.22[a] | 7.03 |
| *P. brassicae* | 6.67 | 1.44 | 56.11[a] | 10.06 |

[a] $P \leq 0.001$ as compared to the assay without MMS. No significant differences within the same column.

(octanal, β-cyclocitral, eugenol, and β-ionone).[8] *P. brassicae* fed with another *B. oleracea* variety (var. *acephala*) was previously described to contain octanal, (−)-menthol, *trans*-geranylacetone, and eugenol.[9] Because plant volatiles are lipophilic substances and can easily cross the cellular membrane, their potential cytotoxicity was evaluated. All tested compounds were cytotoxic at 1 mg/mL, eugenol revealing to be already toxic at 100 μg/mL in the MTT assay (Figure 1). However, at the tested concentrations, the extracts contain these compounds at the pg level or below: volatiles ranged between 0.02 pg for (−)-menthol and 2.20 pg for β-ionone in the larvae aqueous extract and from 0.05 pg of β-cyclocitral to 5.65 pg of eugenol

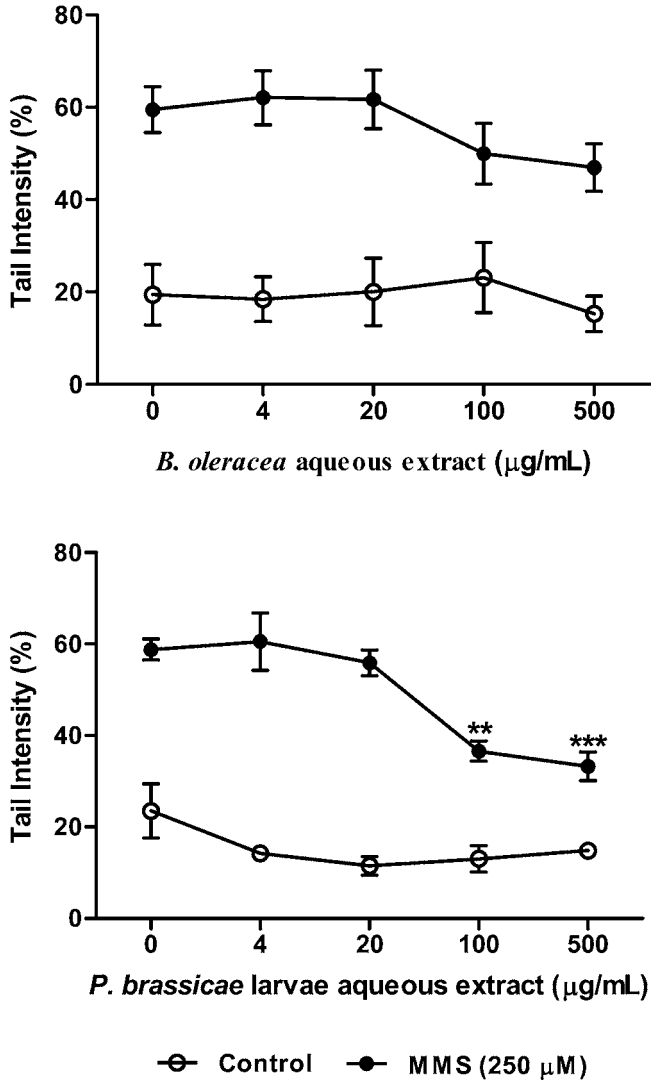


**Figure 3.** Comet tail intensity in V79 cells exposed to the aqueous extracts, performed either with or without MMS. Results are expressed as means ± SEMs. Mean values were significantly different from cells challenged with MMS (**$P < 0.01$, ***$P < 0.001$).

in the *B. oleracea* var. *costata* leaves one, at the highest concentration tested (500 μg/mL). Therefore, no toxicity would be expected for the extracts concerning the amount of volatiles present.

The mutagenic potential of the extracts was screened by a *Salmonella* $His^+$ reversion assay (Ames test) and by the HPRT assay with V79 cells. The Ames test was performed with TA98 strain, which allows the detection of frame-shift mutations.[25] Both extracts proved not to be mutagenic in the Ames assay, with and without an external metabolizing enzyme (Figure 2). Crude juices of *Brassica* vegetables were previously reported to cause genotoxic damage in *Salmonella* TA98 and TA100 strains, without metabolic activation.[26] It is important to refer that the composition of those juices was considerably different from that of the aqueous extracts used in this work. The mutagenic potential of the juices was mainly attributed to isothiocyanates and other glucosinolate breakdown products,[26] the mutagenicity of allyl and phenethyl isothiocyanates being later confirmed.[23] These compounds were not detected in the aqueous extracts assayed herein. However, the aqueous extracts of *P. brassicae* larvae and of *B. oleracea* var. *costata* leaves are rich


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in flavonoids and phenolic acids, as previously reported.[7,10] Such compounds were also considered to contribute to the overall mutagenicity of the *Brassica* vegetables juices, although to a lesser extent than the sulfur compounds.[26] The aqueous extracts of *B. oleracea* var. *costata* leaves and *P. brassicae* larvae fed on them mostly contained highly glycosylated kaempferol derivatives.[7,10] It is known that kaempferol is less mutagenic than the more common flavonol quercetin, the glycosides being considered as nonmutagenic. This can be partly explained by the poor penetration of glycosylated compounds into cells.[27] However, considering human nutrition, the glycosides can be hydrolyzed by bacterial glycosidases present in the lower gut, giving rise to the free aglycone with some mutagenic potential.[28]

It was not possible to evaluate the mutagenic potential of the low molecular weight compounds identified in the aqueous extracts using the Ames test because they exerted antimicrobial activity at low concentrations. This issue was already referred by other authors. For instance, eugenol was reported to be toxic to *S. typhimurium* TA98, TA1537, TA1538, TA100, and TA1535 strains, at 3 mg/plate.[29] In silico screening of mutagenicity to TA98, TA100, and TA1535 strains with the help of predicted activity spectrum for substances (PASS) indicated *n*-octanal to be nonmutagenic, while eugenol was predicted to be mutagenic.[30] $\beta$-Ionone and (−)-menthol at nontoxic concentrations were not mutagenic in the assays with TA100, TA98, TA97a, and TA1535 strains, with and without metabolic activation.[31,32] Furthermore, it was previously found that $\beta$-ionone markedly and dose dependently antagonized the mutagenic effects of aflatoxin B1 and cyclophosphamide, which was thought to be due to the inhibition of CYP2B enzymes.[32] Other authors found that eugenol reduced tobacco-induced mutagenicity in the Ames test.[33] Concerning the volatile composition of the tested extracts, as the amounts of each individual compound were at most in the pg level (Table 1), no mutagenicity was expected.

The mutagenicity of the extracts was also evaluated by the HPRT assay, which is a test widely used to study mutagenicity in mammalian cells, allowing detecting DNA deletions. In this assay, any of the extracts was mutagenic to V79 cells (Table 3). Like in bacterial cells, the breakdown products of glucosinolates are able to induce mutagenicity damage to V79 cells at the HPRT locus,[34] but again, these compounds were not detected in the aqueous extracts. The lack of mutagenicity of the extracts led us to evaluate their potential to act as antimutagenic. MMS, an alkylating agent that induces many different types of adducts in DNA by reaction with its nucleophilic centers, such as nitrogen atoms of DNA bases, was used to induce mutations in V79 cells. Under the experimental conditions used, the extracts did not protect V79 cells against the mutations caused by MMS (Table 3). In fact, although the mutation frequency obtained with the extracts in the assay with MMS was higher than that of the control, particularly in the case of *B. oleracea*, the difference was not significant (Table 3).

The comet assay, which allows evaluation of DNA damage at the cell level, was performed to assess the potential genotoxicity/genoprotection of the extracts. Tail intensity, accounting for the % of damaged DNA, was measured. This parameter was chosen as, in recent years, it has been the recommended end point for the alkaline comet assay. This parameter is the most consistent measurement end point, behaves in a dose-responsive manner, and has defined and comparable units.[35−37]

In this assay, none of the extracts was genotoxic to V79 cells (Figure 3). On the contrary, genoprotection against the genotoxin MMS was observed for the larvae aqueous extract. A tendency to decrease the deleterious effect of MMS was also observed with the *B. oleracea* var. *costata* aqueous extract. It should be emphasized that although the extracts did not reverse the mutagenic effects of MMS at the concentrations necessary to obtain a significant number of HPRT mutants (4 h of exposure was needed to significantly induce mutations, but only 2 h were used to induce genotoxicity in the comet assay), tail intensity showed a tendency to be reduced by exposure to increasing concentrations of the extracts before the MMS challenge. So, the DNA of V79 cells seem to be partly protected by larvae extracts. However, if exposure to MMS significantly induces mutations, the extracts fail to protect the cells, as evaluated by the HPRT assay (Table 3).

The genotoxic/antigenotoxic effects of *Brassica* vegetables seem to depend on the species, test system, doses, duration of the treatment, target tissue, and genotoxic agent, among other factors.[38] Several studies performed with juices rather than aqueous extracts have reported positive results in genotoxicity assays, with the effects being attributed to glucosinolates or their derived products.[26,39] The results are generally more promising when evaluating the antigenotoxic potential of *Brassica* vegetables. For instance, *B. oleracea* var. *italica* juices displayed clastogenic activity in Chinese hamster ovary cells.[40] On the other hand, the protective effects of *Brassica* vegetables against DNA damage induced by heterocyclic amines was reported, both in vivo and in mammalian cells.[12,41] Cotreatment of Hep G2 cells with small amounts of Brussels sprouts juice (0.25−2.0 mL/mL) and B[a]P revealed a reduction of the genotoxic effect of the latter in a dose-dependent manner. In opposition to the protective effect of the crude juice, synergistic effects were observed with allyl isothiocyanate, a breakdown product of sinigrin that is the most abundant glucosinolate in Brussels sprouts, and B[a]P.[42] In another study, the spontaneous rate of mutation of *D. melanogaster* decreased when it was fed with broccoli extracts.[38] The effects of the broccoli diet were dependent on the mechanism of action of the genotoxicant: no effect was observed with a pro-mutagen, synergism was verified with a direct alkylating agent, and modulation of the effect was noticed with a pluripotent carcinogen.[38]

Despite some contradictory results, the consumption of cruciferous vegetables at current levels seems to be beneficial regarding cancer reduction in humans, as concluded before.[26,39] The results obtained in this work can support that *P. brassicae* larvae-derived products can widen the beneficial effects of its host plant. The present data, however, do not provide answers as to the phytochemicals responsible for the antigenotoxic effect. One may speculate that flavonols and hydroxycinnamic acids heterosides, as well as small molecular weight molecules, can contribute to the displayed activity. Among them, eugenol has been thoroughly evaluated for its genotoxic potential in mammalian cells. At 600 $\mu$M, eugenol proved to be genotoxic to some human cell lines (VH10 fibroblasts and Caco-2 colon cells), although it had not the same effect in HepG2 hepatocyte cells.[43] In another work, eugenol induced chromosomal aberrations at 410 $\mu$g/mL in V79 cells and was cytotoxic at higher doses.[44] With S9 activation, the induction of the aberrations was increased in a dose-dependent manner.[44] However, in the aqueous extracts tested herein, the eugenol

concentration is several orders of magnitude below this value (pg).

In conclusion, the aqueous extracts of *B. oleracea* var. *costata* leaves and *P. brassicae* larvae were not mutagenic to bacterial and mammalian cells. Furthermore, the larvae extracts significantly protected V79 cells against the genotoxic effects of MMS, and a tendency to decrease MMS genotoxicity was also observed with the leaves extract. Thus, the previously reported genotoxic potential of crude juices of *Brassica* vegetables[26] could be misleading, since, in general, this kind of vegetables is consumed after prolonged boiling. In addition, as the compounds to which the genotoxic effects of the juices were attributed probably exist in minor amounts (for example, isothiocyanates were not detected in the volatile fraction of the aqueous extracts prepared herein), the consumption of *Brassica* vegetables after a cooking period seems to not constitute a potential risk to DNA. Concerning the *P. brassicae* larvae extract, it can be regarded as a potential source of bioactive compounds, as it was able to protect the DNA in MMS challenged V79 cells.

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### Notes

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