Genetics and Molecular Biology, 37, 1, 93-104 (2014)

www.sbg.org.br

Research Article

Effects of artichoke (*Cynara scolymus*) leaf and bloom head extracts on chemically induced DNA lesions in *Drosophila melanogaster*

Laura Vicedo Jacociunas[1], Rafael Rodrigues Dihl[1,2], Mauricio Lehmann[1,2], Alexandre de Barros Falcão Ferraz[1,2], Marc François Richter[3], Juliana da Silva[1,2] and Heloísa Helena Rodrigues de Andrade[4]

[1]*Programa de Pós-Graduação em Genética e Toxicologia Aplicada, Universidade Luterana do Brasil, Canoas, RS, Brazil.*
[2]*Programa de Pós-Graduação em Biologia Celular e Molecular Aplicada à Saúde, Universidade Luterana do Brasil, Canoas, RS, Brazil.*
[3]*Curso de Biologia Marinha e Costeira, Universidade Estadual do Rio Grande do Sul, Porto Alegre, RS, Brazil.*
[4]*Laboratório de Estomatologia, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.*

Abstract

The genotoxicity of bloom head (BHE) and leaf (LE) extracts from artichoke (*Cynara scolymus* L.), and their ability to modulate the mutagenicity and recombinogenicity of two alkylating agents (ethyl methanesulfonate – EMS and mitomycin C – MMC) and the intercalating agent bleomycin (BLM), were examined using the somatic mutation and recombination test (SMART) in *Drosophila melanogaster*. Neither the mutagenicity nor the recombinogenicity of BLM or MMC was modified by co- or post-treatment with BHE or LE. In contrast, co-treatment with BHE significantly enhanced the EMS-induced genotoxicity involving mutagenic and/or recombinant events. Co-treatment with LE did not alter the genotoxicity of EMS whereas post-treatment with the highest dose of LE significantly increased this genotoxicity. This enhancement included a synergistic increase restricted to somatic recombination. These results show that artichoke extracts promote homologous recombination in proliferative cells of *D. melanogaster*.

Key words: artichoke, *Drosophila melanogaster*, recombinogenicity, SMART.

Received: November 13, 2012; Accepted: August 2, 2013.

Introduction

Artichokes, especially *Cynara scolymus* L. (Asteraceae), have long been consumed as food, especially as a staple component in Mediterranean diets. Several clinical investigations have shown that artichoke extracts can prevent the oxidative modification of blood lipoproteins and reduce blood cholesterol levels (Kirchhoff *et al.*, 1994; Gebhardt, 1998, 2002; Pittler *et al.*, 1998; Zapolska-Downar *et al.*, 2002; Shimoda *et al.*, 2003). Studies of the secondary metabolites of *Cynara* spp. have shown that polyphenolic compounds, mainly caffeic acid derivatives, as well as triterpenoid saponins and flavonoids, play an important biological role in the action of these extracts (Mucaji *et al.*, 1999, 2001).

Send correspondence to Rafael Rodrigues Dihl. Laboratório da Toxicidade Genética e Análise Tóxico-Genética Celular, Universidade Luterana do Brasil, Prédio 22, 4º andar, Avenida Farroupilha 8001, 92425-900 Canoas, RS, Brazil. E-mail: rafael.rodrigues@ulbra.br.

Artichoke leaf extracts (LE) have long been used in traditional folk medicine, mainly because of their choleretic, diuretic and hypocholesterolemic activities (Speroni *et al.*, 2003). Total LE extracts or their constituents reportedly have a beneficial effect in hepato-biliary diseases and improve liver regeneration after partial hepatectomy (Adzet *et al.*, 1987; Kirchhoff *et al.*, 1994; Kraft, 1997; Speroni *et al.*, 2003). These extracts also have antioxidative and protective properties against hydroperoxide-induced oxidative stress in cultured rat hepatocytes (Gebhardt and Fausel, 1997; Miccadei *et al.*, 2004). The central part of the artichoke flower bud is the edible portion of the plant and is widely consumed in Spain (2.6 g/day/person) (MAPA, 2003). Extracts of *Cynara cardunculus* L. (ECC) significantly reduced the frequency of 4-nitroquinoline-N-oxide-induced revertants at the *ilv1* locus and mitotic gene convertants at the *trp5* locus in the diploid *Saccharomyces cerevisiae* strain D7 (Miadokova *et al.*, 2008). An anticlastogenic effect against N-nitroso-N'-methylurea in *Vicia sativa* L (Miadokova *et al.*, 2008) and against ethyl

methanesulfonate (EMS) in *Drosophila melanogaster* has also observed (Miadokova *et al.*, 2006). In contrast, although ECC is not mutagenic in *Salmonella typhimurium* TA98, it significantly increased the mutagenic effect of 2-aminofluorene and enhanced the cytotoxic/cytostatic effect of cis-Pt (Miadokova *et al.*, 2006). We have also recently demonstrated the genotoxic and antigenotoxic potential of *C. scolymus* L. leaf extract *in vitro* (Jacociunas *et al.*, 2012, 2013).

The aim of this study was therefore: (1) to characterize the mutagenic and recombinogenic activity of artichoke bloom head (BHE) and leaf (LE) extracts, and (2) to explore the antigenotoxic potential of a combination of both extracts against chemical agents capable of inducing distinct types of DNA lesions in eukaryotes. The bioassay used was the Somatic Mutation and Recombination Test (SMART) in D. melanogaster. This test allows assessment of the potential of complex mixtures to cause a loss of heterozygosity in marker genes of somatic cells, expressed as mutation and somatic recombination. Two genetic markers, *multiple wing hair* (*mwh*) and *flare* (*flr*^{3}) in the third chromosome, were used to detect local recombinogenic effects linked toeuchromatin and the heterochromatin centromeric region (Graf *et al.*, 1984).

Material and Methods

Plant extracts

The *C. scolymus* L. specimens used in this work were collected in Gramado (Rio Grande do Sul, Brazil), on a small farm in Apiquárius (latitude 29º27.851' and longitude 50º49.501') where the plants were organically cultivated. The artichoke leaves and flowers were collected in the winter of 2007 and a voucher specimen was deposited in the Herbarium of the Department of Botany of the Lutheran University of Brazil (HERULBRA 4288).

Preparation of extracts

Crude aqueous extracts of leaves (120 g) and flowers (160 g) were prepared by infusion with distilled water (plant:solvent ratio of 1:10) at 80 °C for 30 min. The infusion was cooled at room temperature, filtered, frozen and concentrated by lyophilization. The resulting yields were 13.7 g (11.4%) for leaf extracts and 14.8 g (9.3%) for flowers.

The phytochemical profiles of the extracts were determined as described by Harbone (1998) and Simões *et al.* (2007). These methods involve colorimetric reactions that qualitatively detect flavonoids, tannins, saponins, alkaloids, anthraquinones, coumarins and cardiac glycosides. The presence of saponins, flavonoids and coumarins was also analyzed by thin-layer chromatography (TLC) in silica gel GF254 using eluents and developers indicated by Wagner and Bladt (1996). The phytochemical screening of LE and BHE identified the presence of flavonoids, phenolic compounds and saponins.

Chemicals

The chemical compounds ethylmethanesulphonate (EMS, 62-50-0), liquid form M0880, was obtained from Sigma Chemical Co. (Saint Louis, MO, USA). The bifunctional agent mitomycin C (MMC, 50-07-5) was obtained from Bristol-Myers Squibb (São Paulo, SP, Brazil). Bleomycin sulfate (BLM – 9041-93-4) was obtained from Biossintética (São Paulo, SP, Brazil). These agents and the extracts were dissolved in distilled water immediately before use.

Somatic mutation and recombination test (SMART) in *D. melanogaster* crosses

Two versions of the SMART were used: (1) standard (ST) cross: *flr*^{3}/ TM3, *Bd*^{S} females to *mwh/mwh* males and (2) high bioactivation (HB) cross: *ORR/ORR; flr*^{3}/ TM3, *Bd*^{S} females to *mwh/mwh* males (Graf and van Schaik, 1992). Eggs from the two crosses were collected for 8 h on standard medium enriched with baker's yeast and supplemented with sucrose. After three days, the third instar larvae were washed out of the vials and used for the treatments.

Genotoxicity test

Chronic treatments (from 48 h until pupation) were done by adding similar-aged larvae (72 ± 4 h) from the ST and HB crosses to vials containing 1.5 g of *Drosophila* Instant Medium (Carolina Biological Supply Company, Burlington, NC, USA) plus 3 mL of fresh BHE (0.0069, 0.0138, 0.0276 and 0.0552 g/mL) or LE (0.01875, 0.02175, 0.0435 and 0.0875 g/mL), previously diluted in distilled water. The toxicity of these extracts was assessed in a pilot experiment in which batches of 100 flies were treated with different concentrations of each extract. The number of surviving flies was counted and at least 70% of the flies reached the adult stage in all treatments. The extracts were tested in triplicate in two independent experiments. Distilled water was used as a negative control.

Co-treatment

Larvae from the ST cross were transferred to plastic vials containing 1.5 g of *Drosophila* Instant Medium rehydrated with 3 mL of the test solution containing distilled water alone, mutagenic compound, or extract (LE: 0.0435 and 0.0875 g/mL; BHE: 0.0276 and 0.0552 g/mL) plus the mutagen (12.5 mM EMS; 0.5 mM MMC or 0.01 mM BLM). The larvae were left to feed and then complete development on this medium. MMC, BLM or EMS alone were used as positive controls and distilled water alone was the negative control (Andrade *et al.*, 2004; Sinigaglia *et al.*, 2006).

Post-treatment

Larvae from the ST cross were transferred to Plexiglas vials, the lower end of which was covered with fine nylon gauze. These tubes were then placed in 50 mL beakers containing 0.3 g of powered cellulose (Merck) mixed with 2 mL of distilled water or different mutagen solutions. The larvae were fed on these mutagen-cellulose suspensions through the gauze for 4 h (for EMS and BLM) and 6 h (for MMC). The groups (subjected to acute feeding with water or genotoxin) were then washed and put into plastic vials with 1.5 g of *Drosophila* Instant Medium containing either distilled water or different concentrations of the extracts (0.0435 g/mL and 0.0875 g/mL). The larvae were allowed to feed on the instant medium until pupation (± 48 h).

Wing scoring

Approximately 10-12 days after the treatments, the emerging adult flies were collected and conserved in 70% ethanol. The *mwh* x *flr*^{3} standard cross produced two types of progeny that were distinguished phenotypically based on the *Bd*^{s} marker: (1) trans-heterozygous flies for the recessive wing-cell markers *multiple wing hair (mwh)* and *flare (flr*^{3}*)* and (2) heterozygous flies for a balancer chromosome with large inversions on chromosome 3 (TM3). Wings of five females and five males of the two phenotypes were mounted on slides and scored under a microscope at 400X magnification for the occurrence of spots. Induced loss of heterozygosity in the marker-heterozygous genotype leads to two types of mutant clones: (1) single spots, either *mwh* or *flr*^{3}, that result from point mutations, chromosomal aberrations and/or somatic recombination, and (2) twin spots, consisting of both *mwh* and *flr*^{3} sub-clones, that originate exclusively from somatic recombination (Graf *et al.*, 1984). In flies with the balancer-heterozygous genotype, *mwh* spots reflect predominantly somatic point mutations and chromosomal aberrations since somatic recombination involving the balancer chromosome and its structurally normal homologue is a non-viable event. By comparing the frequencies of these two genotypes it was possible to quantify the modulatory effect of *C. scolymus* L. on the recombinogenic and mutagenic activities of the genotoxins (Frei *et al.*, 1992).

Statistical analysis

The data were evaluated according to the multiple-decision procedure of Frei and Würgler (1988), which produces four possible diagnoses: positive, weakly positive, negative or inconclusive. The frequencies of each type of mutant clone per fly of a treated series were compared pair-wise, *i.e.*, control *vs.* modulator; genotoxin alone *vs.* genotoxin plus modulator, using the conditional binomial test according to Kastenbaum and Bowman (1970). All inconclusive and weak results were analyzed with the non-parametric U-test of Mann, Whitney and Wilcoxon. The U-test takes into account the rank values in controls and treatments and considers over-dispersion in a non-normal distribution (Frei and Würgler, 1995). For both tests $p < 0.05$ was considered significant. Based on the control-corrected frequencies of clone formation per 10^5 cells, the percentages of modulator interference were calculated as follows: [(genotoxin alone - genotoxin plus modulator/genotoxin alone) X 100] (Abraham, 1994).

Results

Genotoxicity

The genetic toxicity analyses of both BHE and LE were done in the ST and HB crosses by observing the occurrence of clone spot induction in marker-trans-heterozygous (*mwh/flr*^{3}) adult flies. For each concentration used, Tables 1 and 2 show the total number of flies analyzed, the frequency of the different mutant clones and the total spots scored, which represent the final genotoxicity of the extracts tested. For all four doses used, neither extract showed a significant difference in relation to the respective negative controls in the ST and HB crosses, which means they were clearly not genotoxic in this test system.

MMC, BLM and EMS were genotoxic and produced somatic recombination in marker-heterozygous (*mwh/flr*^{3}) flies (Table 3). Likewise, significant mutational responses were observed; each of the compounds increased the frequency of total spots in balancer-heterozygous (*mwh*/TM3) flies. The frequencies of mutant spots induced by EMS in the later genotype were smaller than those obtained in trans-heterozygous flies (Tables 4 and 5). These findings are consistent with previously reported responses for these compounds in the SMART assay (Sinigaglia *et al.*, 2004, 2006). In addition, the genotoxicity of BLM was preferentially related to the induction of small single and total spots, as previously described by Graf *et al.* (1984).

Modulator effects

Since MMC, EMS and BLM act as direct genotoxins the modulatory effects of both extracts was analyzed only in the ST cross. In the co-treatment protocol and in the trans-heterozygous genotype, neither BHE nor LE modified the MMC and BLM spot frequencies, which suggested that neither extract interfered with the mechanisms that precede the induction of DNA lesions by these genotoxins. Conversely, there was a significant increase in the frequency of mutant clones in response to EMS for both concentrations of BHE, but not for LE (Tables 3 and 4). In the balancer-heterozygous genotype (TM3), BHE also significantly increased the frequencies of total spots induced by EMS (by ~60 and 130% for 0.0276 and 0.0552 g BHE/mL, respectively), indicating that the extract was both co-mutagenic and co-recombinogenic (Table 4). Figure 1 shows the synergistic effect of co-treatment with BHE on EMS genotoxicity, particularly in relation to mutation and recombination. EMS alone (12.5 mM) induced 41.2 spots through

Table 1 - Genotoxicity of leaf extracts (LE) from *Cynara scolymus* L. in the *D. melanogaster* wing spot test using standard (ST) and high bioactivation (HB) crosses.

Crosses / genotypes	LE (g/mL)	No. of flies (N)	Spots per fly (no. of spots)/statistical diagnosis [a]				Total *mwh* clones[c] (n)	Mean *mwh* clone size class[c]	Clone induction frequencies (per 10^5 cells per cell division)[d, e] (n/NC)[f]
			Small single spots[b] (1-2 cells) (*m* = 2)	Large single spots[b] (> 2 cells) (*m* = 5)	Twin spots (*m* = 5)	Total spots[b] (*m* = 2)			
ST cross									
mwh / flr^3	NC[g]	40	0.95 (38)	0.08 (03)	0.03 (01)	1.05 (42)	42	1.71	2.15
	0.01875	40	0.50 (20) -	0.08 (03) -	0.10 (04) -	0.68 (27) -	27	2.04	1.38 -[0.77]
	0.02175	40	0.63 (25) -	0.05 (02) -	0.03 (01) -	0.70 (28) -	28	1.89	1.43 -[0.72]
	0.0435	40	0.45 (18) -	0.08 (03) -	0.00 (00) -	0.53 (21) -	21	1.76	1.08 -[1.08]
	0.0875	40	0.68 (27) -	0.05 (02) -	0.05 (02) -	0.78 (31) -	31	1.68	1.59 -[0.56]
HB cross									
mwh / flr^3	NC[g]	40	0.93 (37)	0.10 (04)	0.00 (00)	1.03 (41)	41	1.76	2.10
	0.01875	40	0.70 (28) -	0.13 (05) -	0.05 (02) -	0.88 (35) -	34	1.88	1.74 -[0.36]
	0.02175	40	0.83 (33) -	0.10 (04) -	0.00 (00) -	0.93 (37) -	37	1.81	1.90 -[0.20]
	0.0435	40	0.55 (22) -	0.13 (05) -	0.08 (03) -	0.75 (30) -	29	2.28	1.49 -[0.61]
	0.0875	40	0.70 (28) -	0.08 (03) -	0.00 (00) -	0.78 (31) -	30	1.67	1.54 -[0.56]

[a]Statistical diagnoses according to Frei and Würgler (1988, 1995). Two-tailed U-test: -, negative; *m*: minimal risk multiplication factor for the assessment of negative results; significance levels $\alpha = \beta = 0.05$; [b]Including rare *flr*^{3} single spots; [c]Considering *mwh* clones from *mwh* single and twin spots; [d]Numbers in square brackets are induction frequencies corrected for spontaneous incidence estimated from negative controls; [e]For calculation see Andrade *et al.* (2004); [f]C = 48.800, *i.e.*, approximate number of cells examined per fly; [g]Negative control (NC).

Table 2 - Genotoxicity of bloom heads extracts (BHE) from *Cynara scolymus* L. in the *D. melanogaster* wing spot test using standard (ST) and high bioactivation (HB) crosses.

Crosses / genotypes	BHE (g/mL)	No. of flies (N)	Spots per fly (no. of spots)/statistical diagnosis[a]				Total *mwh* clones[c] (n)	Mean *mwh* clone size class[c]	Clone induction frequencies (per 10^5 cells per cell division)[d, e] (n/NC)[f]
			Small single spots[b] (1-2 cells) (*m* = 2)	Large single spots[b] (> 2 cells) (*m* = 5)	Twin spots (*m* = 5)	Total spots[b] (*m* = 2)			
ST cross									
mwh / flr^3	NC[g]	40	0.70 (28)	0.13 (05)	0.03 (01)	0.85 (34)	34	1.65	1.74
	0.0069	40	0.65 (26) -	0.05 (02) -	0.05 (02) -	0.75 (30) -	30	1.63	1.54 - [0.20]
	0.0138	40	0.55 (22) -	0.10 (04) -	0.03 (01) -	0.68 (27) -	26	2.00	1.33 - [0.41]
	0.0276	40	0.50 (20) -	0.20 (08) -	0.08 (03) -	0.78 (31) -	31	2.65	1.59 - [0.15]
	0.0552	40	0.53 (21) -	0.13 (05) -	0.10 (04) -	0.75 (30) -	30	2.30	1.54 - [0.20]
HB cross									
mwh / flr^3	NC[g]	40	0.95 (38)	0.03 (01)	0.08 (03)	1.05 (42)	42	1.57	2.15
	0.0069	40	0.88 (35) -	0.15 (06) -	0.05 (02) -	1.08 (43) -	43	1.82	2.25 [0.10]
	0.0138	40	0.80 (32) -	0.05 (02) -	0.13 (05) -	0.98 (39) -	39	1.72	2.00 - [0.15]
	0.0276	40	0.95 (38) -	0.08 (03) -	0.00 (00) -	1.03 (41) -	41	1.61	2.10 [0.05]
	0.0552	40	0.73 (29) -	0.10 (04) -	0.03 (01) -	0.85 (34) -	34	1.74	1.74 - [0.41]

[a]Statistical diagnoses according to Frei and Würgler (1988, 1995). Two-tailed U-test: -, negative; *m*: minimal risk multiplication factor for the assessment of negative results; significance levels $\alpha = \beta = 0.05$; [b]Including rare *flr*^{3} single spots; [c]Considering *mwh* clones from *mwh* single and twin spots; [d]Numbers in square brackets are induction frequencies corrected for spontaneous incidence estimated from negative controls; [e]For calculation see Andrade *et al.* (2004); [f]C = 48.800, *i.e.*, approximate number of cells examined per fly; [g]Negative control (NC).

Table 3 - Summary of results obtained in the *D. melanogaster* wing spot test. Co-treatments with MMC, BLM and EMS in combination with leaf extracts (LE) from *Cynara scolymus* L., 48 h feeding of 3-day-old larvae of the standard (ST) cross: marker-trans-heterozygous flies (*mwh/flr*^{3}).

Genotypes	Controls and compounds MUT[b] + LE (g/mL)	No. of flies (N)	Spots per fly (no. of spots)/statistical diagnosis [a]				Total *mwh* clones[d] (n)	Mean *mwh* clone size class[d]	Clone induction frequencies (per 10^5 cells per cell division)[e, f] (n/NC)[g]
			Small single spots[c] (1-2 cells) (*m* = 2)	Large single spots[c] (> 2 cells) (*m* = 5)	Twin spots (m = 5)	Total spots[c] (*m* = 2)			
	MMC								
mwh / flr^3	NC[h]	40	0.65 (26)	0.15 (06)	0.00 (0)	0.80 (32)	32	2.00	1.64
	MMC	40	34.30 (1372)*	31.17 (1247)*	13.53 (541)*	79.00 (3160)*	3003	2.90	153.84 [152.20]
	MMC + 0.0435	40	33.73 (1349) -	29.30 (1172) -	12.75 (510) -	75.78 (3031) -	2588	2.94	132.58 [130.94]
	MMC + 0.0875	40	32.62 (1305) -	29.93 (1197) -	13.20 (528) -	75.76 (3030) -	2480	2.57	131.26 [129.62]
	BLM								
mwh / flr^3	NC[h]	30	0.70 (21)	0.10 (03)	0.10 (03)	0.90 (27)	27	1.81	1.84
	BLM	30	3.07 (92)*	0.53 (16)*	0.03 (01)	3.63 (109)*	109	1.85	7.45 [5.60]
	BLM + 0.0435	30	2.50 (75) -	0.27 (08) -	0.10 (03) -	2.87 (86) -	86	1.70	5.87 [4.03]
	BLM + 0.0875	30	2.76 (83) -	0.27 (08) -	0.00 (00) -	3.03 (91) -	91	1.66	6.22 [4.37]
	EMS								
mwh / flr^3	NC[h]	30	0.70 (21)	0.10 (03)	0.10 (03)	0.90 (27)	27	1.81	1.84
	EMS	30	93.63 (2809)*	36.60 (1098)*	21.60 (648)*	151.83 (4555)*	4336	2.25	296.17 [294.33]
	EMS+ 0.0435	30	100.37 (3011) -	34.07 (1022) -	14.40 (432) +	148.84 (4465) -	4397	2.22	293.34 [291.35]
	EMS + 0.0875	30	105.43 (3163) +	26.93 (808) +	17.27 (518) +	149.63 (4489) -	4378	2.22	299.04 [294.20]

[a]Statistical diagnoses according to Frei and Würgler (1988, 1995). Two-tailed U-test: *, positive; $p \leq 0.05$ *vs.* untreated control; +, positive and -, negative, $p \leq 0.05$ *vs.* MMC, BLM or EMS alone; *m*: minimal risk multiplication factor for the assessment of negative results; [b]Mutagen: MUT; [c]Including rare *flr*^{3} single spots; [d]Considering *mwh* clones from *mwh* single and twin spots; [e]Numbers in square brackets are induction frequencies corrected for spontaneous incidence estimated from negative controls; [f]For calculation see Andrade *et al.* (2004); [g]C = 48.800, *i.e.*, approximate number of cells examined per fly; [h]NC = negative control.

Table 4 - Summary of results obtained in the *D. melanogaster* wing spot test. Co-treatments with MMC, BLM and EMS in combination with bloom heads extracts (BHE) from *Cynara scolymus* L., 48 h feeding of 3-day-old larvae of the standard (ST) cross: marker-trans-heterozygous flies (*mwh/flr*^{3}) and balancer-heterozygous (*mwh*/TM3) flies.

Genotypes	Controls and compounds MUT[b] + BHE (g/mL)	No. of flies (N)	Spots per fly (no. of spots)/statistical diagnosis[a]				Total *mwh* clones[d] (n)	Mean *mwh* clone size class[d]	Clone induction frequencies (per 10^5 cells per cell division)[e, f] (n/NC)[g]	Enhancement[h] (%)
			Small single spots[c] (1-2 cells) (*m* = 2)	Large single spots[c] (> 2 cells) (*m* = 5)	Twin spots (*m* = 5)	Total spots[c] (*m* = 2)				
	MMC									
mwh / flr^3	NC[i]	40	0.65 (26)	0.15 (06)	0.00 (0)	0.80 (32)	32	2.00	1.64	
	MMC	40	34.30 (1372)*	31.18 (1247)*	13.53 (541)*	79.01 (3160)*	3003	2.90	153.84 [152.20]	
	MMC + 0.0276	40	35.03 (1401) -	32.55 (1302) -	14.75 (590) -	82.33 (3293) -	3137	2.89	160.71 [159.07]	
	MMC + 0.0552	40	33.33 (1333) -	32.25 (1290) -	14.08 (563) -	79.66 (3186) -	3053	2.94	156.40 [154.76]	
	BLM									
mwh/ flr^3	NC[i]	40	0.73 (29)	0.13 (05)	0.07 (03)	0.93 (37)	37	1.76	1.90	
	BLM	40	3.20 (128)*	0.40 (16)*	0.03 (01)	3.63 (145)*	144	1.73	7.38 [5.48]	
	BLM + 0.0276	40	3.65 (146) -	0.20 (08) -	0.10 (04) -	3.95 (158) -	158	1.66	8.09 [6.20]	
	BLM + 0.0552	40	2.80 (112) -	0.20 (08) -	0.03 (01) -	3.03 (121) -	121	1.70	6.20 [4.30]	
	EMS									
mwh / flr^3	NC[i]	30	0.70 (21)	0.10 (03)	0.10 (03)	0.90 (27)	27	1.81	1.84	
	EMS	30	93.63 (2809)*	36.60 (1098)*	21.60 (648)*	151.83 (4555)*	4336	2.25	296.17 [294.33]	
	EMS + 0.0276	30	138.83 (4165) +	48.53 (1456) +	28.83 (865) +	216.19 (6486) +	6233	2.08	425.75 [423.91]	44.02
	EMS + 0.0552	30	159.30 (4779) +	47.43 (1423) +	39.97 (1199) +	246.70 (7401) +	6901	2.00	471.38 [469.54]	59.53
mwh / TM3	NC[i]	30	0.47 (14)	0.00 (0)	[j]	0.47 (14)	14	1.21	0.96	
	EMS	30	33.53 (1006)*	5.70 (171)*		39.23 (1177)*	1177	1.54	80.40 [79.44]	
	EMS+ 0.0276	30	53.67 (1610) +	9.00 (270) +		62.67 (1880) +	1880	1.57	128.42 [127.46]	60.45
	EMS + 0.0552	30	78.27 (2348) +	11.37 (341) +		89.64 (2689) +	2689	1.52	183.67 [182.72]	130.01

[a]Statistical diagnoses according to Frei and Würgler (1988, 1995). Two-tailed U-test: *, positive; $p \leq 0.05$ *vs.* untreated control; +, positive and -, negative, $p \leq 0.05$ *vs*. MMC, BLM or EMS alone; *m*: minimal risk multiplication factor for the assessment of negative results; significance levels $\alpha = \beta = 0.05$; [b]Mutagen: MUT; [c]Including rare *flr*^{3} single spots; [d]Considering *mwh* clones from *mwh* single and twin spots; [e]Numbers in square brackets are induction frequencies corrected for spontaneous incidence estimated from negative controls; [f]For calculation see Andrade *et al.* (2004); [g]C = 48.800, *i.e.*, approximate number of cells examined per fly; [h]Calculated according to Abraham (1994) using the control corrected clone induction frequencies: (MUT alone - MUT plus BHE / MUT alone) x 100; [i]Negative control: NC; [j]Only *mwh* single spots were observed in *mwh*/TM3 heterozygotes as the balancer chromosome TM3 does not carry the *flr*^{3} mutation.

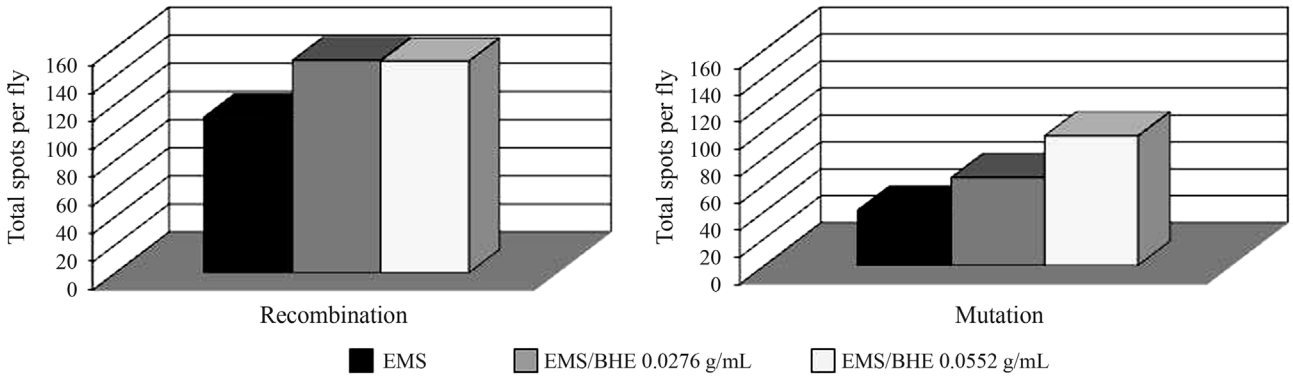

Figure 1 - Contribution of mutation and recombination to the frequency of total spots per fly in trans-heterozygous flies treated with EMS in combination (co-treatment) with BHE (0.0276 and 0.0552 g/mL). The recombinogenic activity was calculated according to Sinigaglia *et al.* (2004, 2006) as follows: Mutation frequency (F_M) = frequency of spots in balancer-heterozygous/frequencies of spots in marker-trans-heterozygous. Recombination frequency (F_R) = 1-F_M. Frequency of total spots (F_T) = total spots in *mwh*/*flr*^{3} flies (considering *mwh* and *flr*^{3} spots)/number of flies. Mutation = F_T X F_M. Recombination = F_T X F_R.

mutational events and 110.6 related to somatic recombination. BHE (0.0276 and 0.0552 g/mL) increased the mutagenic activity of EMS to 65.2 and 96.1 spots, respectively (increments of 1.6 and 2.3 fold). In contrast, BHE (0.0276 and 0.0552 g/mL) had only a minor effect on EMS recombinogenicity (~151 spots, 1.4 fold increase for both concentrations). These results for the co-genotoxicity of BHE with EMS and the lack of effect on the genotoxicity of BLM and MMC probably reflect differences in the mechanisms of action of alkylating agents (MMC and EMS) compared to the intercalating drug BLM.

In the post-treatment protocols, BHE did no significantly affect the genotoxicity of the agents tested. Likewise, LE did not interfere with the mutagenic and recombinogenic action of MMC and BLM. These data indicate that post-treatment with both extracts had no effect on the mechanisms involved in the MMC- and BLM-induced lesions (Tables 5 and 6). The outcome of LE on EMS--induced activity was quite different since this extract significantly increased the frequency of EMS-induced spots by 131% at the highest dose tested. These effects were observed solely in *mwh*/*flr*^{3} flies since in *mwh*/TM3 flies post-treatment with LE did not alter the frequency of EMS-induced spots (Table 5).

Discussion

The non-mutagenic and recombinogenic effect of artichoke BHE and LE was demonstrated in the wing SMART assay in a standard cross of *D. melanogaster* (basal metabolism) and in a high bioactivation cross (HB). The metabolic differences between the two crosses reflect variation in their cytochrome P450 (CYP450) levels. The ORR-flare strain has chromosomes 1 and 2 from a DDT-resistant Oregon R(R) line, that contribute to high levels of CYP450. In particular, the CYP6A2 level is increased, primarily as a result of a mutation of the CYP450 regulatory gene *Rst(2)DDT*. Our data indicate the absence of direct and indirect BHE- and LE-mediated mutagenic and recombinogenic activities. Only one report in the literature has examined the genotoxicity of *C. scolymus* L. and found that leaf and flower extracts did not induce chromosomal mutation in peripheral blood and bone narrow cells, as assessed by the micronucleus test; these extracts were also not genotoxic in the Comet assay, except at the highest concentration of leaf extract (2000 mg/kg) (Zan MA, 2008, MSc dissertation, Universidade Luterana do Brasil, Porto Alegre, Brazil). *Cynara cardunculus* is also not mutagenic in the Ames test and *Saccharomyces cerevisiae* assay, and not clastogenic in *Vicia sativa* (Miadokova *et al.*, 2008).

The usefulness of SMART for studying antigenotoxic effects is reinforced by the finding that some modulators that decrease the incidence of mutational effects are equally able to increase the occurrence of somatic recombination. This means that modulating agents should be evaluated not only in terms of their action on mutagenic events (point and chromosomal mutations), but also in relation to their effects on somatic recombination. Because trans-heterozygous flies express all of these genetic endpoints, SMART offers an additional advantage over other assays in that it allows one to establish the pharmacological behavior of modulating agents, as described earlier (Santos *et al.*, 1999; Sinigaglia *et al.*, 2004, 2006).

In the co-treatment protocols, neither BHE nor LE modified the frequencies of MMC- or BLM-mutant spots, indicating that neither extract interfered with the steps that precede the DNA-induced lesions, such as antioxidant activity, the suppression of metabolic activation and the stimulation of detoxification via the induction of glutathione S-transferase (Aboobaker *et al.*, 1994; Morse *et al.*, 1995).

Since BLM and MMC can induce oxidative damage (Cederberg and Ramel, 1989; Povirk and Austin, 1991; Tomasz, 1995), we may infer that the mixture represented for LE and BHE had no scavenger activity to prevent drug-induced oxidative damage. Phytochemical analyses

Table 5 - Summary of results obtained in the *D. melanogaster* wing spot test. Acute exposure to MMC (6 h), BLM and EMS (4 h) followed by post-treatment with leaf extracts (LE) from *Cynara scolymus* L., 3-day-old standard (ST) cross larvae: marker-trans-heterozygous (*mwh/flr*^{3}) and balancer-heterozygous (mwh/TM3) flies.

Genotypes	Controls and compounds MUT[b] + LE (/g/mL)	No. of flies (N)	Spots per fly (no. of spots)/statistical diagnosis [a]				Total mwh clones[d] (n)	Mean *mwh* clone size class[d]	Clone induction frequencies (per 10^5 cells per cell division)[e, f] (n/NC)[g]	Enhancement[h] (%)
			Small single spots[c] (1-2 cells) (*m* = 2)	Large single spots[c] (> 2 cells) (*m* = 5)	Twin spots (*m* = 5)	Total spots[c] (*m* = 2)				
	MMC									
mwh / flr^3	NC[i]	40	0.75 (30)	0.08 (03)	0.08 (03)	0.91 (36)	36	1.69	1.84	
	MMC	40	1.78 (71)*	7.78 (311)*	2.63 (105)*	12.18 (487)*	440	4.73	22.54 [20.70]	
	MMC + 0.0435	40	1.50 (60) -	8.05 (322) -	3.05 (122) -	12.60 (504) -	468	4.61	23.98 [22.13]	
	MMC + 0.0875	40	1.28 (51) -	8.80 (352) -	3.38 (135) -	13.45 (538) -	504	4.69	25.82 [23.98]	
	BLM									
mwh / flr^3	NC[i]	30	0.70 (21)	0.10 (03)	0.07 (02)	0.87 (26)	26	1.96	1.78	
	BLM	30	1.74 (52)*	0.23 (07)	0.03 (01)	2.00 (60)*	58	1.67	3.96 [2.19]	
	BLM + 0.0435	30	1.87 (56) -	0.17 (05) -	0.03 (01) -	2.07 (62) -	62	1.68	4.23 [2.46]	
	BLM + 0.0875	30	1.83 (55) -	0.20 (06) -	0.00 (00) -	2.03 (61) -	61	1.80	4.17 [2.39]	
	EMS									
mwh / flr^3	NC[i]	30	0.63 (19)	0.03 (01)	0.00 (0)	0.66 (20)	20	1.90	1.37	
	EMS	30	3.30 (99)*	3.17 (95)*	2.40 (72)*	8.87 (266)*	224	2.79	15.30 [13.93]	
	EMS + 0.0435	30	4.57 (137) -	3.80 (114) -	2.37 (71) -	10.74 (322) -	277	2.62	18.92 [17.55]	
	EMS + 0.0875	30	7.73 (232) +	6.67 (200) +	5.50 (165) +	19.90 (597) +	492	2.69	33.61 [32.24]	131.44
mwh / TM3	NC[i]	30	0.47 (14)	0.00 (0)	[j]	0.47 (14)	14	1.29	0.96	
	EMS	30	1.90 (57)*	0.83 (25)*		2.73 (82)*	82	2.06	5.60 [4.64]	
	EMS + 0.0875	30	2.40 (72) -	1.00 (30) -		3.40 (102) -	102	2.11	6.97 [6.01]	

[a]Statistical diagnoses according to Frei and Würgler (1988, 1995). Two-tailed U-test: *, positive; $p \leq 0.05$ *vs.* untreated control; +, positive and -, negative, $p \leq 0.05$ *vs*. MMC, BLM or EMS alone; *m*: minimal risk multiplication factor for the assessment of negative results; significance levels $\alpha = \beta = 0.05$; [b]Mutagen: MUT; [c]Including rare *flr*^{3} single spots; [d]Considering *mwh* clones from *mwh* single and twin spots; [e]Numbers in square brackets are induction frequencies corrected for spontaneous incidence estimated from negative controls; [f]for calculation see Andrade *et al.* (2004); [g]C = 48.800, *i.e.*, approximate number of cells examined per fly; [h]Calculated according to Abraham (1994) using the control corrected clone induction frequencies: (MUT alone - MUT plus LE / MUT alone) x 100; [i]Negative control: NC; [j]Only *mwh* single spots were observed in *mwh*/TM3 heterozygotes as the balancer chromosome TM3 does not carry the *flr*^{3} mutation.

Table 6 - Summary of results obtained in the *D. melanogaster* wing spot test. Acute exposure to MMC (6 h), BLM and EMS (4 h) followed by post-treatment with bloom heads extracts (BHE) from *Cynara scolymus* L., 3-day-old standard (ST) cross larvae: marker-trans-heterozygous (*mwh/flr*^{3}).

Genotypes	Controls and compounds MUT[b] + BHE(g/mL)	No. of flies (N)	Spots per fly (no. of spots)/statistical diagnosis [a]				Total mwh clones[d] (n)	Mean *mwh* clone size class[d]	Clone induction frequencies (per 10^5 cells per cell division)[e, f] (n/NC)[g]
			Small single spots[c] (1-2 cells) (*m* = 2)	Large single spots[c] (> 2 cells) (*m* = 5)	Twin spots (*m* = 5)	Total spots[c] (*m* = 2)			
	MMC								
mwh / flr^3	NC[i]	50	0.72 (36)	0.10 (05)	0.10 (05)	0.92 (46)	46	1.86	1.89
	MMC	50	2.40(120)*	8.12 (406)*	2.62 (131)*	13.14 (657)*	604	4.52	24.75 [22.87]
	MMC + 0.0276	50	1.36 (68) +	8.02 (401) -	3.12 (156) -	12.50 (625) -	592	4.90	24.26 [22.38]
	MMC + 0.0552	50	2.08 (104) -	7.92 (396) -	2.74 (137) -	12.74 (637) -	601	4.55	24.63 [22.75]
	BLM								
mwh / flr^3	NC[i]	30	0.70 (21)	0.10 (03)	0.07 (02)	0.87 (26)	26	1.96	1.78
	BLM	30	1.72 (52)*	0.23 (07)	0.03 (01)	2.00 (60)*	58	1.67	3.96 [2.19]
	BLM + 0.0276	30	1.36 (41) -	0.23 (07) -	0.03 (01) -	1.63 (49) -	49	2.33	3.35 [1.57]
	BLM + 0.0552	30	2.13 (64) -	0.10 (03) -	0.03 (01) -	2.26 (68) -	68	1.68	4.64 [2.87]
	EMS								
mwh / flr^3	NC[i]	30	0.63 (19)	0.03 (01)	0.00 (0)	0.66 (20)	20	1.90	1.37
	EMS	30	3.30 (99)*	3.17 (95)*	2.40 (72)*	8.87 (266)*	224	2.79	15.30 [13.93]
	EMS + 0.0276	30	2.87 (86) -	3.30 (99) -	1.60 (48) +	7.77 (233) -	207	2.89	14.14 [12.77]
	EMS + 0.0552	30	4.00 (120) -	4.37 (131) -	2.40 (72) -	10.77 (323) -	268	2.75	18.31 [16.94]

[a]Statistical diagnoses according to Frei and Würgler (1988, 1995). Two-tailed U-test: *, positive; $p \leq 0.05$ *vs.* untreated control; +, positive and -, negative, $p \leq 0.05$ *vs.* MMC, BLM or EMS alone; *m*: minimal risk multiplication factor for the assessment of negative results; significance levels $\alpha = \beta = 0.05$; [b]Mutagen: MUT; [c]Including rare *flr*^{3} single spots; [d]Considering *mwh* clones from *mwh* single and twin spots; [e]Numbers in square brackets are induction frequencies corrected for spontaneous incidence estimated from negative controls; [f]For calculation see Andrade *et al.* (2004); [g]C = 48.800, *i.e.*, approximate number of cells examined per fly; [h]Calculated according to Abraham (1994) using the control corrected clone induction frequencies: (MUT alone - MUT plus BHE / MUT alone) x 100; [i]Negative control: NC.

of both extracts have identified flavonoids, such as chlorogenic acid, that act as antioxidants and pro-oxidants (Cao *et al.*, 1997). However, Sotibrán *et al.* (2011) demonstrated that flavonoids, including chlorogenic acid, are unable to induce oxidative stress in *D. melanogaster* nor protect DNA against paraquat-induced oxidative stress lesions. A similar behavior was also observed in the post-treatment protocol. Conversely, in the co-treatment experiments, both concentrations of BHE significantly increased the frequency of mutant clones in response to EMS in the trans-heterozygous genotype. In TM3 balancer-heterozygous flies there were significant increases in the total number of spots indicative of co-recombinogenic and/or co-mutagenic activities. In this genotype, spots originate exclusively by mutational events since recombination produced unviable configurations (because of multiple-inversions in the heterozygous state of TM3 balancer chromosomes) (Graf *et al.*, 1984).

The increase in spots seen in balancer-heterozygous individuals indicated that co-treatment with BHE affected both endpoints, which were more related to EMS-mutational events. However, the highest LE concentration used in the post-treatment protocols also exerted a synergistic effect against EMS in trans-heterozygous flies (~ 131% increase), but not in TM3 flies. These findings indicate that the synergistic recombinogenic activity of the LE extract was related to the type of lesions induced and, consequently, to the repair processes, *e.g.*, homologous recombination (HR), involved in their correction. It is unclear why LE modulates the genotoxicity of EMS (which is preferentially associated with damage caused by N-alkylation damage) and O6-ethyldeoxyguanosine.

Our results indicate that the modulatory action of both extracts was quite different since the synergistic effects on EMS-mediated genotoxicity was restricted to somatic recombination in the case of LE and preferentially associated with mutation in the case of BHE, at least in *Drosophila* proliferative cells.

There are no reports on the modulatory effect of *Cynara* extracts against MMC and BLM. Extract of *C. cardunculus* (ECC) showed a specific protective effect on yeast cells undergoing mutagenic and convertogenic changes induced by 4-nitroquinoline-*N*-oxide, and also reduced the anticlastogenic effect of N-nitroso-*N*-methylurea in *Vicia sativa* in co-treatment experiments. However, this extract significantly increased the mutagenic effect of 2-aminofluorene in *Salmonella typhimurium* TA98 (Miadokova *et al.*, 2008). This finding correlates well with the results of Ogawa *et al.* (1987), who observed a flavonoid-mediated increase in the mutagenicity of 2-acetylaminofluorene (2-AAF) in the presence of rat liver microsomes. On the other hand, ECC reduced the genotoxicity of EMS in the sex-linked recessive lethal mutation (SLRL) in *D. melanogaster* via the inactivation of EMS (Miadokova *et al.*, 2006).

LE and BHE contained flavonoids, phenolic compounds and saponins. The major flavonoids present in artichoke florescences include narirutin (Wang *et al.*, 2003), apigenin (Zhu *et al.*, 2004) and cyanidin (Schutz *et al.*, 2006), whereas the main constituents of leaves are luteolin and luteolin glycosides (Noldin *et al.*, 2003, Wang *et al.*, 2003). In addition to flavonoids, the phenolic acids described as leaf constituents include caffeic acid and ferulic acid (Noldin *et al.*, 2003), cynarin and chlorogenic acid (Speroni *et al.*, 2003), also present in florescences. Although apigenin and luteolin have antimutagenic activity (Birt *et al.*, 1986; Czeczot *et al.*, 1990; Duthie *et al.*, 2000; Romanova *et al.*, 2001) these compounds are also mutagenic and clastogenic in a variety of eukaryotes and *in vivo* systems (Ogawa *et al.*, 1987; Snyder and Gillies, 2002).

Based on the findings reported here, we suggest that each extract contains a unique complex mixture that can increase the frequency of genotoxic events induced by EMS. The increase in EMS-mediated recombination must be associated with different mechanisms, including interference in the steps that precede EMS-induced genotoxicity and in the mechanisms involved in correcting EMS-specific damage.

Homologous somatic recombination may result in a loss of heterozygosity or genetic rearrangements, and these events are involved in the genesis of numerous diseases, including cancer (Bishop and Schiestl, 2003). It would be interesting to determine which components in the extracts are responsible for the synergistic effects on EMS genotoxicty and their interference on other genotoxic agents.

Acknowledgments

This work was supported by Lutheran University of Brazil (ULBRA), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

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Associate Editor: Daisy Maria Fávero Salvadori