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Modulation of hepatic ABC transporters by *Eruca vesicaria* intake: Potential diet-drug interactions

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ABSTRACT

The aim of this work was to evaluate whether oral administration of *Eruca vesicaria*, a species of rocket cultivated in Argentina, could modify cyclophosphamide (CP)-induced genotoxicity through modulation of hepatic ABC transporters. Daily oral administration of *E. vesicaria* fresh leaves juice (1.0, 1.4 and 2.0 g/kg) for 14 days did not alter genotoxicity biomarkers —alkaline comet assay and micronucleus test —in neither male nor female mice. Instead, repeated intake of this cruciferous decreased CP-induced DNA damage dose-dependently and it caused hepatic overexpression of P-glycoprotein (P-gp; 1.4 and 2.0 g/kg) and multidrug resistance protein 2 (MRP2; 2.0 g/kg), but not breast cancer resistance protein (Bcrp). The antigenotoxic effect of *E. vesicaria* was prevented by 50 mg/kg verapamil (P-gp inhibitor) or 10 mg/kg indomethacin (MRP2 inhibitor). In turn, CP-induced cytotoxicity (10 mM, 24 h) on human hepatoma cells (HepG2/C3A) was significantly reduced by preincubation with *E. vesicaria* (1.4 mg/ml; 48 h); this effect was absent when CP was coincubated with 35 μM verapamil, 80 μM indomethacin or 10 μM KO-143 (BCRP inhibitor).

Altogether, these results allow us to demonstrate that repeated intake of *E. vesicaria* exhibited antigenotoxicity, at least in part, by induction of hepatic ABC transporters *in vivo* in mice as well as *in vitro* in human liver cells. This could account for other diet-drug interactions.

1. Introduction

The widespread use of herbal medicines, nutraceuticals and dietary natural products concomitantly with pharmacotherapy has raised concerns on their possible interactions and, therefore, effect on drugs efficacy. Herbs and dietary ingredients can alter transport or metabolic processes, affecting drug disposition (Fugh-Berman, 2000; Izzo and Ernst, 2009).

The cultivated plant *Eruca vesicaria*—common name "rocket" —is an edible cruciferous vegetable (Brassicaceae family) whose consumption has increased in the last ten years in Argentina. Several *cruciferae* extracts or their isolated phytochemicals, such as sulforaphane, erucin and erysolin, have shown protective activity against DNA damage inducers both *in vitro* and *in vivo* (Jin et al., 2009; Lamy et al., 2008; Sharma et al., 2016). On the other hand, it has also been described that fractions rich in isothiocyanates and other glucosinolate-breakdown products from several Brassicaceae vegetables can cause DNA damage and clastogenicity in mammalian cells (Baasanjav-Gerber et al., 2011; Fimognari et al., 2012; Kassie et al., 1996; Kupke et al., 2016; Wiesner et al., 2014). Both deleterious and beneficial effects of isolated compounds of *cruciferae* were associated with tissue-specific modulation of cytochrome P450 and phase II conjugation systems in several *in vitro* and *in vivo* models of cancer (Canistro et al., 2012; Higdon et al., 2007; Konsue and Ioannides, 2008; Paolini et al., 2003; Perocco et al., 2006; Plate and Gallaher, 2006).

However, the induction of hepatic enzymes caused by glucosinolates only partially reproduced the potent antigenotoxic effect of black cabbage seeds or *Eruca sativa* leaf juice on human hepatoma cells (HepG2) (Canistro et al., 2012; Lamy et al., 2008). This supports the theory that several phytochemicals would act synergistically through

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different mechanisms of action to achieve the beneficial effect.

In this sense, phytochemicals found in cruciferous extracts and juices, including *E. vesicaria,* interact with ATP Binding Cassette (ABC) transporters (Aszalos, 2008; Baiceanu et al., 2015; Dewanjee et al., 2017; Ebert et al., 2006; Miron et al., 2017; Telang et al., 2009). ABC transporters are involved in cell detoxification (Xu et al., 2005) by expeling xenobiotics or endogenous waste products against concentration gradients in an active ATP-dependent way (Efferth and Volm, 2017). P-glycoprotein (P-gp/MDR1/ABCB1), multidrug resistance protein 2 (MRP2/ABCC2 or its murine homologue Mrp2) and breast cancer resistance protein (BCRP/ABCG2 or its murine homologue Bcrp) are ABC transporters expressed in the canalicular membrane of hepatocytes, which pour their substrates into the bile (Schinkel and Jonker, 2003). Expression and activity of ABC transporters vary between individuals due to sex, genetic polymorphisms, pathological conditions, xenobiotic exposure or dietary habits, leading to differences in bioavailability of different drugs, toxins or food-derived carcinogens (Lu and Klaassen, 2007; Patel et al., 2016; Suzuki et al., 2006). Previous studies from our work group demonstrated that watercress and wild rocket (*Diplotaxis tenuifolia*) exhibit a protective effect against genotoxic damage induced by the alkylating agent cyclophosphamide (CP) (Casanova et al., 2013; Casanova and Carballo, 2011; López Nigro et al., 2018). CP is an indirect mutagen metabolized by hepatic cytochrome P450 system to several metabolites including the active 4-hydroxycyclophosphamide (OH-CP) that circulates in blood and enters cells as the tautomer aldophosphamide (Fleming, 1997). In turn, aldophosphamide is transformed into phosphoramide mustard and acrolein, which react with the DNA forming adducts and inter and intra-strand cross-links (Fleming, 1997; Hemminki, 1987; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). Liver phase II enzymes, such as glutathione transferase, associated with ABC transporters like Mrp2 that dump their conjugated products into bile, are essential for the elimination of CP and its metabolites in rat liver (Qiu et al., 2003). Interactions between ABC transporters and CP or its metabolites in other tissues have also been reported for BCRP, MRP4 and P-gp (Brayboy et al., 2013; Zhang et al., 2005). In addition, we have observed that *D. tenuifolia* intake modulates Bcrp as well as Mrp2 in mouse liver (López Nigro et al., 2018).

Thus, the modulation of ABC transporters by natural product compounds could affect the bioavailability of both CP and its active metabolites. The goal of the present study was to determine whether chronic intake of *E. vesicaria* has the ability to modulate hepatic ABC efflux transporters and the relevance of this interaction for its genotoxic or protective effect *in vivo* in mice as well as *in vitro* in human hepatic cells.

2. Materials and methods

2.1. Chemicals

Methanol (purity > 99%), Giemsa solution, modified May-Grünwald's eosine-methylene blue solution, NaCl (purity > 99%), dimethylsulfoxide (DMSO, purity > 99%) and NaOH (purity > 99%) were purchased from Merck (Argentina). Ethidium bromide (purity > 95%), acridine orange (purity > 95%), Na_2EDTA (purity > 99%), Trizma Base (purity > 99%), Triton X-100, Tween 20, Cyclophosphamide, phenylmethanesulfonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), verapamil, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1′,2′:1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester hydrate (Ko-143), indomethacin, trypan blue, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Fetal bovine serum, phosphate buffered saline, low-melting point agarose (LMPA) and regular agarose were purchased from Gibco (Waltham, Massachusetts, U.S.A.). Heparin was from Abbott (Illinois, U.S.A). Protease inhibitor cocktail (Complete mini) was purchased from Roche Applied Science (Mannheim, Germany).

2.2. Preparation and phytochemical screening of Eruca vesicaria juice

E. vesicaria leaves were obtained from certified organic gardens and processed according to Lopez Nigro et al. (López Nigro et al., 2018). Briefly, leaves were washed and dried and the stalks were removed prior to weighting. The remaining plant material (310.6 g) was processed with a commercial juice maker, and the juice was afterwards centrifuged (14,000 rpm for 20 min at 4 °C). The supernatant (122 ml) was clarified, filter-sterilized (0.22 μm) and diluted according to experimental requirements. The juice was kept on ice and away from the light throughout the entire procedure. Small aliquots were frozen at −80 °C until use.

Subsequently, we performed a quantitative phytochemical screening of some of the main active components of the juice. For the assessment of total phenols, the Folin-Ciocalteu method was used (Makkar et al., 1993). Absorbance was recorded at 725 nm and gallic acid was used as a standard for the calibration curve. Total flavonoids were determined by adding aluminium chloride and sodium acetate to aliquots of the juice and recording the absorbance at 430 nm after room temperature incubation (Maksimović et al., 2005). A rutin calibration curve was used for quantification. Lastly, the modified Dao and Friedman method was performed for the measurement of hydroxycinnamic acids (Dao and Friedman, 1992). Aliquots of 50 μl of the juice were diluted in 2 ml ethanol and absorbance was measured at 328 nm, using a chlorogenic acid calibration curve for quantification.

2.3. Animals

Male and female Swiss mice (7–8 weeks old, 25–30 g body weight) were housed under 12:12 h light:dark cycles, at standard temperature (20–25 °C) and humidity (60 ± 10%) conditions, with food and water *ad libitum*. A total number of 64 animals were randomly divided into 8 groups per sex containing 4 males and 4 females per group in the animal house facility at ININFA (Instituto de Investigaciones Farmacológicas – Universidad de Buenos Aires–CONICET). All procedures involving animals were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) and Argentinian regulation for animal facilities (Administración Nacional de Medicamentos Alimentos y Tecnología Médica, 1996).

2.3.1. Juice doses and animal treatment

E. vesicaria juice doses were selected on the basis of an approximate daily consumption of 70, 100 and 140 g of rocket salad for an average adult, these being equivalent to one to two servings per day according to the guidelines (Hartmann et al., 2003; MacGregor et al., 1987). Male and female mice were treated according to Lopez Nigro et al. (López Nigro et al., 2018), as shown in Fig. 1. Briefly, animals were weighted

Fig. 1. Treatment scheme. CP: cyclophosphamide, i.p.: intraperitoneal injection.

daily and a dose of 1.0, 1.4 or 2.0g/kg body weight of rocket juice for each treatment group were administered by gavage in a volume of 0.01ml/g body weight for 14 consecutive days. Two groups representing the negative and positive controls received a similar volume dose of saline solution (0.9% NaCl).

On day 15, half of the groups of mice received an intraperitoneal injection (i.p.) of 50 mg/kg of CP (Positive Control and DNA Protection groups), the other half received an i.p. injection of saline solution (Negative Control and Genotoxicity groups). On day 16, all mice were sacrificed by cervical dislocation to obtain heparinized blood, both femora and the liver.

To evaluate the role of ABC transporter modulation on *E. vesicaria* antigenotoxicity, an additional group of 24 female mice was randomly divided into 6 groups of 4 animals each. Only females were used since no differences between genders were evidenced in neither genotoxicity nor ABC transporter expression for the highest *E. vesicaria* juice dose. All mice were weighted daily and received a dose of 2 g/kg body weight of *E. vesicaria* juice for 14 consecutive days. On day 15, they received i.p. injections of: a) Group 2.0 g/kg + SS: saline solution; b) Group 2.0 g/kg + CP: 50 mg/kg CP; c) Group 2.0 g/kg + V: 50 mg/kg verapamil; d) Group 2.0 g/kg + V + CP: 50 mg/kg verapamil and 50 mg/kg CP; e) Group 2.0 g/kg + I: 10 mg/kg indomethacin; and f) Group 2.0 g/kg + I + CP: 10 mg/kg indomethacin and 50 mg/kg CP. Doses of ABC transporter inhibitors were selected on the basis of reported evidences (Dahan and Amidon, 2010; Horton, 1989).

On day 16, mice were sacrificed by cervical dislocation and both femora were collected.

2.4. Hepatic expression of ABC efflux transporters

2.4.1. Preparation of liver membranes

Isolated livers from the negative and positive controls, and 1.0, 1.4, 2.0 g/kg *E. vesicaria* groups (n = 4 per group) were collected, rinsed in cold saline and promptly stored at −70 °C. Fraction enriched in plasma membrane was prepared by differential centrifugation after homogenization in 0.3 M sucrose containing 0.1 mM phenylmethylsulfonyl fluoride, 25 mg/ml leupeptin, 5 mg/ml aprotinin and 5 mg/ml pepstatin A (50 mg liver per ml of buffer) (López Nigro et al., 2018).

2.4.2. Western blot analysis

Protein concentration in homogenates was measured (Lowry et al., 1951) with BSA as standard. Proteins (30 μg/well) were separated on a 7.5% SDS-polyacrylamide gel and transferred to a poly(vinylidene difluoride) (PVDF) membrane (Amersham Hybond GE Healthcare Life Sciences, UK). The membranes were blocked with 5% nonfat milk prepared in TBS-Tween 20 and were afterwards cut at molecular mass 52 and 102 kDa, based on Full-Range Rainbow Molecular Weight Markers (GE Healthcare Life Sciences, UK). For detection of ABC transporter proteins, each portion of membrane was incubated with primary antibodies as follows: mouse monoclonal anti MDR1 (anti P-gp; ABCB1; D-11; IgG2b; 1:500; Santa Cruz Biotechnologies; CA, USA), mouse monoclonal anti ABCG2 (anti Bcrp, BXP-21; IgG2a; 1:800; Santa Cruz Biotechnologies; CA, USA) and mouse monoclonal anti ABCC2 (anti Mrp2; M2III-5; IgG2b; 1:150; Abcam; Cambridge, UK), diluted in 1% serum albumin in TBS-Tween 20. As load control, rabbit polyclonal anti-actin (1:1000; Sigma-Aldrich) was used. Goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (sc-2004; 1:2000; Santa Cruz Biotechnologies) and goat anti-mouse horseradish peroxidase-conjugated immunoglobulin G (sc-2005; 1:2000; Santa Cruz Biotechnologies) were used as secondary antibodies, respectively. Chemiluminescence reaction (Amersham ECL Biosciences, Little Chalfont, UK) was used to visualize bands and optical densitometry was performed with NIH ImageJ free software. Assays were carried out in duplicate.

2.5. In vivo assessment of the genotoxic and/or DNA protective properties of *Eruca vesicaria*

2.5.1. Alkaline comet assay in mice blood cells

The comet assay (Single Cell Gel Electrophoresis) was performed in its alkaline version, following the protocol by Singh et al. with modifications (Singh et al., 1988). The animals were sacrificed by cervical dislocation, and blood was rapidly extracted through cardiac puncture with a heparinized syringe. Great care was taken in order to avoid direct light exposure of the samples throughout the entire procedure. The Mercille and Massie fluorescent method (Mercille and Massie, 1994), which uses ethidium bromide and acridine orange to differentiate living and dead cells, was used to assess cell viability of the samples. Only samples that showed cell viability greater than 80% were considered adequate for this test.

Microscope slides were coated with a layer of 1% regular agarose. Afterwards, the blood samples were mixed with 0.5% low melting-point agarose at 37 °C and placed on top, with the addition of a final layer of low melting-point agarose. These slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% dimethylsulfoxide and 1% Triton-X100, pH 10) and kept refrigerated overnight. Subsequently, DNA unwinding was carried out with cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.5) for 20 min, generating single-strand DNA thus allowing the expression of alkali-labile sites and single-strand breaks. Following this step, electrophoresis was performed on a horizontal electrophoresis system (BioRad, Hercules, CA, USA) using electrophoresis buffer at a constant voltage of 0.7 V/cm and 300 mAmp for 20 min at 4 °C. Finally, the gels were neutralized with Tris 0.4 M, pH 7.5 and the slides were stained with 20 μg/ml ethidium bromide. The analysis was performed on an Olympus BX40 fluorescence microscope. For each sample, 100 comets in duplicate were scored and classified into four damage categories according to DNA migration, and a Damage Index (DI, in arbitrary units) was calculated for each sample as follows: $DI = n_1 + 2xn_2 + 3xn_3 + 4xn_4$ (where n_x represent the number of comets in categories 1 to 4). In addition, Damage Reduction Indices (DRIs) were obtained (Serpeloni et al., 2008): $Reduction\ (\%) = \frac{DI\ C+ - DI\ X}{DI\ C+ - DI\ C-} \times 100$ whereC + represents the mean of the positive control group, X represents the genotoxicity observed in the mice that received *E. vesicaria* juice plus CP and C- represents the mean of the negative control group.

2.5.2. In vivo mice micronucleus test

The protocol for this assay was adapted from Schmid (1975). Both femuri were extracted and washed with saline solution. The epiphyses were cut, and the medullar canal was washed with a syringe containing fetal bovine serum and a 25G needle. The bone marrow cell suspension obtained was centrifuged and resuspended in fetal bovine serum. Slides were prepared by cytocentrifugation, air-dried and fixed with methanol. May-Grünwald/Giemsa staining was performed for the identification of polychromatic (PCE) and normochromatic (NCE) erythrocytes by their differential coloration. Five hundred erythrocytes were scored in order to obtain the Cytotoxicity Index: PCE/(PCE + NCE), and 1000 PCE and NCE were scored in duplicate for the assessment of the frequency of micronuclei in 2000 PCE and NCE for each sample. A Reduction Index was determined (Serpeloni et al., 2008) in an analogous way to the comet assay:

$$Reduction\ (\%) = \frac{MicronucleiPCE\ C + - MicronucleiPCE\ X}{MicronucleiPCE\ C + - MicronucleiPCE\ C-} \times 100$$

2.6. In vitro cell viability assays in human liver cells

2.6.1. Cell culture

Human hepatic carcinoma cells C3A [HepG2/C3A, derivative of HepG2 (ATCC HB¬8065)] (ATCC® CRL¬10741™) were kindly provided

by Dr. María Fernanda Troncoso (Espelt et al., 2019). Cells were cultured in Dulbecco's MEM medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin at 37 °C and 5% CO_2 in a humidified incubator.

2.6.2. Cell treatment with E. vesicaria juice, CP and ABC inhibitors

Cells were seeded in DMEM complete medium on 48 or 96-well microplates (Greiner Bio-One) and cultured for 24 h. A concentration-response curve to CP (1–25 mM) showed that 10 mM CP caused a 50% decrease in cell viability for the MTT assay as well as the Trypan Blue exclusion test after 24 h. Following pretreatment for 48 h with *E.vesicaria* (1.4 mg/ml) or its vehicle (saline solution), cells were further exposed to 10 mM CP for 24 h alone or in the presence of verapamil (35 μM), KO-143 (10 μM, BCRP inhibitor) or indomethacin (80 μM). Culture medium was then replaced by fresh complete DMEM and cells were cultured for further 24 h.

2.6.3. MTT assay

HepG2/C3A cells were seeded on 96-well microplates and treated as described in section 2.6.2. Once the treatment was completed, medium was removed, and cells were washed with PBS. Fresh medium containing 0.45 mg/ml MTT was added to each well and cells were incubated for 3 more hours for MTT reduction by living cells. Formazan crystals thus formed were dissolved in 100 μl of isopropanol at acid pH. Absorbance at 570 nm and 660 nm (as a reference) were measured with a microplate spectrophotometer (FlexStation 3 Multi-Mode Microplate Reader). Results are expressed as the mean percentage of cell viability of four independent experiments.

2.6.4. Trypan blue exclusion test

HepG2/C3A cells were seeded in 48 well-plates and treated as described in 2.6.2. After trypsinization, cells were diluted in the same volume of 0.5% trypan blue dye and the percentage of viable cells was recorded by observation with a Neubauer hemocytometer on an optical microscope (Nikon Eclipse) equipped with fluorescent optics. Results were expressed as the mean percentage of cell viability of four independent experiments.

2.7. Statistical analysis

Experimental data is expressed as mean ± SD and the statistical analysis was performed using IBM's SPSS software. Genotoxicity and cell viability assays were analyzed by one way ANOVA, Kruskal-Wallis, post-hoc Dunnett's test for multiple comparisons, t-test and Mann-Whitney's U test. Immunoblotting analysis was performed using Kruskal-Wallis and post-hoc Dunn's test for multiple comparisons.

3. Results

3.1. Quantitative phytochemical screening of Eruca vesicaria leaves juice

The results of the quantitative analysis of *E. vesicaria* juice (2.55 g/ml) are presented in Table 1.

Table 1
Quantitative phytochemical analysis of *E. vesicaria* juice (2.55 g/ml).

	Concentration (mean ± SD)
Total Flavonoids (μg rutin/mL)	65.82 ± 1.35
Hydroxycinnamic acid (μg chlorogenic acid/mL)	310.69 ± 10.88
Total Phenols (μg gallic acid/mL)	526.0 ± 21.43

3.2. Effect of chronic intake of E. vesicaria leaves juice on hepatic expression of ABC transporters

Animal average weight gain as well as food and water consumption showed no significant differences between all the groups at the end of the treatment ($p > 0.05$).

As shown in Fig. 2, in control conditions, the protein expression of P-gp (Fig. 2A) and Mrp2 (Fig. 2B) was significantly higher in females compared to males ($p < 0.05$ and $p < 0.01$, respectively) whereas no sex-related differences were observed for the hepatic expression of Bcrp (Fig. 2C; $p > 0.05$). Moreover, no modifications on the expression profile of hepatic ABC transporters were found after 24 h of a single administration of CP (50 mg/kg, i.p.; $p > 0.05$), as shown in Fig. 2.

With respect to the animal groups that received *E. vesicaria* juice, a significant increase in protein abundance of hepatic P-gp was observed in male (Fig. 3A) and female (Fig. 3D) mice treated with 1.4 mg/kg ($p < 0.05$) and 2.0 mg/kg ($p < 0.01$), remaining unchanged for the lowest dose ($p > 0.05$). Additionally, a significant increase in Mrp2 was observed only for the highest dose (2.0 g/kg) in livers from both male and female mice (Fig. 3B and E; $p < 0.05$). On the other hand, no changes were observed on the expression of Bcrp for either sex (Fig. 3C and F; $p > 0.05$).

3.3. Genotoxicity and DNA protective effect of E. vesicaria leaves juice

No significant differences on genotoxicity biomarkers among male and female mice from the same treatment group ($p < 0.05$) were found, hence data from both genders were pooled.

The comet assay showed that the DNA damage (expressed by the Damage Index in arbitrary units) of mice that received daily doses of *E. vesicaria* juice during 14 days was not significantly different from those receiving saline solution (Fig. 4A; $p > 0.05$). On the contrary, mice that received the rocket juice treatment and afterwards a CP injection evidenced significantly lower DNA damage than those that did not received *E.vesicaria* juice (Fig. 4A; $p < 0.001$) for the three tested doses.

Accordingly, the frequency of Mn in bone marrow PCE was not different between saline solution and *E. vesicaria* juice-treated mice ($p > 0.05$). Nonetheless, there was a significant reduction in PCE MN frequency in mice that received the 3 doses of the juice (1.0, 1.4 and 2.0 g/kg) and subsequently the CP injection in contrast with those that did not receive the juice treatment ($p < 0.05$, Fig. 4B).

Furthermore, the Cytotoxicity Index (CI) allowed us to assess a potential cytotoxic effect which may interfere with the maturation kinetics of the red blood cells precursors. No differences were observed when the CI was compared between all the groups tested (Table 2; $p > 0.05$).

Additionally, the frequency of micronuclei in NCE was recorded to test genotoxicity prior to the administration of the DNA damage inducer CP (basal genotoxicity). All the groups evidenced frequencies comparable to those of negative controls ($p > 0.05$), suggesting that all mice had undergone no previous genotoxic exposures (Table 2).

The Damage Reduction Indices (DRIs) for the comet assay and the Mn test calculated for 1.0, 1.4 and 2.0 g/kg E. *vesicaria* juice were the following: a) for the comet assay: 61.80%, 72.76% and 79.27%; b) for the Mn test: 9.45%, 35.32% and 43.28%. Oral administration of the cruciferous was associated with a dose-dependent increase in DRIs for both DNA damage biomarkers. Moreover, the *E. vesicaria*-induced increase in DRIs was higher for the comet assay than for the Mn test at each tested dose.

3.4. Effects of in vivo inhibition of ABC transporters on E. vesicaria antigenotoxicity

Following the same treatment scheme, we analyzed the role of ABC transporters in the antigenotoxic effect of 2.0 g/kg *E.vesicaria,* which causes hepatic overexpression of P-gp and Mrp2 (see section 3.2.). The

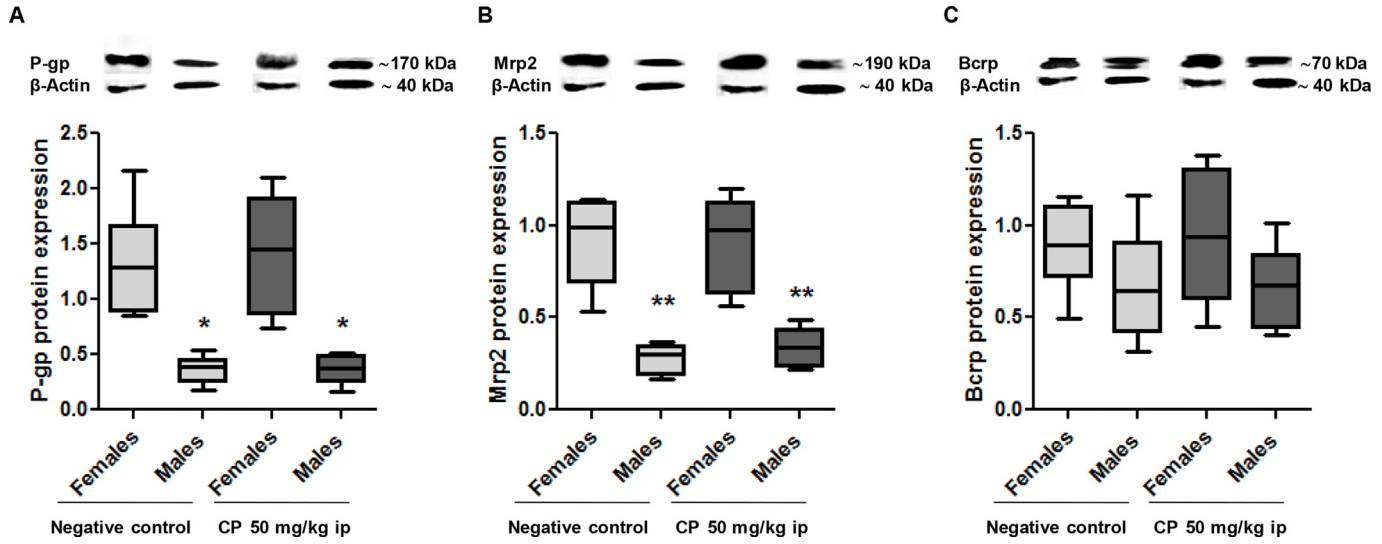

Fig. 2. Effect of a single dose of CP on hepatic ABC transporter expression. Representative immunoblots of P-gp (A), Mrp2 (B), and Bcrp (C) in liver of adult male and female mice. Animals treated with saline solution during 14 days received an i.p. injection of saline solution (Negative control, light gray boxes) or 50 mg/kg body weight (dark gray boxes) on day 15. Densitometric units were calculated as ratio between optical density of the ABC transporter and the load control band. Results are expressed as median and the lower and upper quartiles of four mice per group. *$p < 0.05$ and **$p < 0.01$ between male and female for P-gp and Mrp2 expression, respectively, in the corresponding treatment (Kruskall Wallis with Dunn's post-hoc test).

mice micronucleus test was selected for genotoxicity assessment due to its robustness and the correlation between this biomarker and the comet assay that we observed in the first group of mice (Fig. 4A and B).

As shown in Fig. 5, no differences in the frequency of Mn in PCE were found between the group of animals that received only *E. vesicaria* juice and those that also received the P-gp inhibitor (50 mg/kg verapamil; $p > 0.05$) or the Mrp2 inhibitor (10 mg/kg indomethacin; $p > 0.05$). In accordance with results presented in section 3.3, mice that received CP following the juice treatment showed a significant increase of this biomarker (Fig. 5; $p < 0.05$).

Interestingly, mice that received *E. vesicaria* juice, CP and verapamil or indomethacin evidenced a marked increase in the frequency of Mn, in contrast with those that were exposed to the juice and CP only (Fig. 5; $p < 0.001$).

Regarding CI and the frequency of Mn in NCE, no differences were found among any of the groups ($p > 0.05$, data not shown).

3.5. Effect of hepatic ABC transporter inhibition on the protective action of *E. vesicaria* in human hepatocytes

Finally, to further analyze the involvement of hepatic ABC transporters in the protective effect of *E. vesicaria*, an *in vitro* experimental set was carried out in human hepatocellular carcinoma cells, which display xenobiotic metabolism and ABC transporter pathways (HepG2/C3A). Cell viability was examined by two complementary assays: MTT assay (Fig. 6A) and Trypan Blue exclusion test (Fig. 6B). Selected concentrations of ABC inhibitors were not cytotoxic *per sé*. As shown in Fig. 6A and B, exposure to 1.4 mg/ml E. *vesicaria* juice did not affect cell viability. Nevertheless, 10 mM CP for 24 h produced a decrease of approximately 50% in cell viability respect to the negative control, but pretreatment with *E. vesicaria* juice significantly reduced CP-induced cytotoxicity (Fig. 6A and B; $p < 0.05$). However, the protective effect of *E. vesicaria* juice was prevented when the P-gp inhibitor (35 μM verapamil; Fig. 6A and B; $p < 0.001$) was co administered with CP. Similarly, coadministration of CP with the MRP2 inhibitor (80 μM indomethacin; Fig. 6A and B; $p < 0.001$) significantly reverted the protective effect of the cruciferous. Interestingly, the BCRP inhibitor (10 μM Ko-143), also avoided the protective effect of *E.vesicaria* leaves juice on CP-induced cytotoxicity (Fig. 6A and B; $p < 0.01$).

4. Discussion

The present study showed that repeated oral administration of fresh leaves of *E. vesicaria* has a protective effect against genotoxicity caused by CP. This effect would depend, at least in part, on the overexpression of the hepatic efflux transporters P-gp and Mrp2 caused by sustained exposure to this cruciferous in mice. Moreover, an anti-cytotoxic effect of *E.vesicaria* dependent on P-gp, MRP2 and BCRP was also observed *in vitro* in human liver cells.

The alkaline comet assay in blood lymphocytes and the Mn test in mice bone marrow are genotoxicity effect biomarkers frequently used in a complementary way to detect different types of DNA damage (Bowen et al., 2011; Kang et al., 2013). The comet assay, or single-cell gel electrophoresis, detects DNA-strand breaks in virtually any type of cells including blood cells. The absence of modifications in the DNA damage index (DI) for the comet assay after the repeated ingestion of *E. vesicaria* leaf juice, showed that the crucifer did not cause any breaks or alkali-labile sites in the DNA of mice leucocytes. Regarding the *in vivo* Mn test, during erythropoiesis, lagging chromosomes or acentric fragments in the cytoplasm of dividing red blood cells precursors give rise to smaller nuclei after telophase, which are known as micronuclei (MacGregor et al., 1987; Schmid, 1975). These remain in the cytoplasm of polychromatic erythrocytes, which are formed once the erythroblast expels its main nucleus. The fact that the MN frequencies were not increased in mice treated with *E. vesicaria* juice suggests that this cruciferous is not clastogenic or aneugenic at the doses used. Additionally, only micronuclei in PCE are originated from a recent genotoxic insult, whereas micronuclei in NCE are indicative of previous damage (Mavournin et al., 1990; Schmid, 1975). According to this, we could corroborate that erythropoietic cells suffered no cytotoxic damage since PCE and NCE ratio (Cytotoxicity Index) was comparable among all groups. All together, these results show that repeated oral intake of *E. vesicaria* was not genotoxic in doses comparable to human average intake.

Cyclophosphamide toxicity is based on the high reactivity of the nitrogen mustard —one of its metabolites, generated through cytochrome P450— which interacts with guanine residues forming inter and intrastrand cross-links. This process leads to inhibition of DNA synthesis and cytotoxicity (Baumann and Preiss, 2001). These characteristics made cyclophosphamide an extremely suitable agent to be

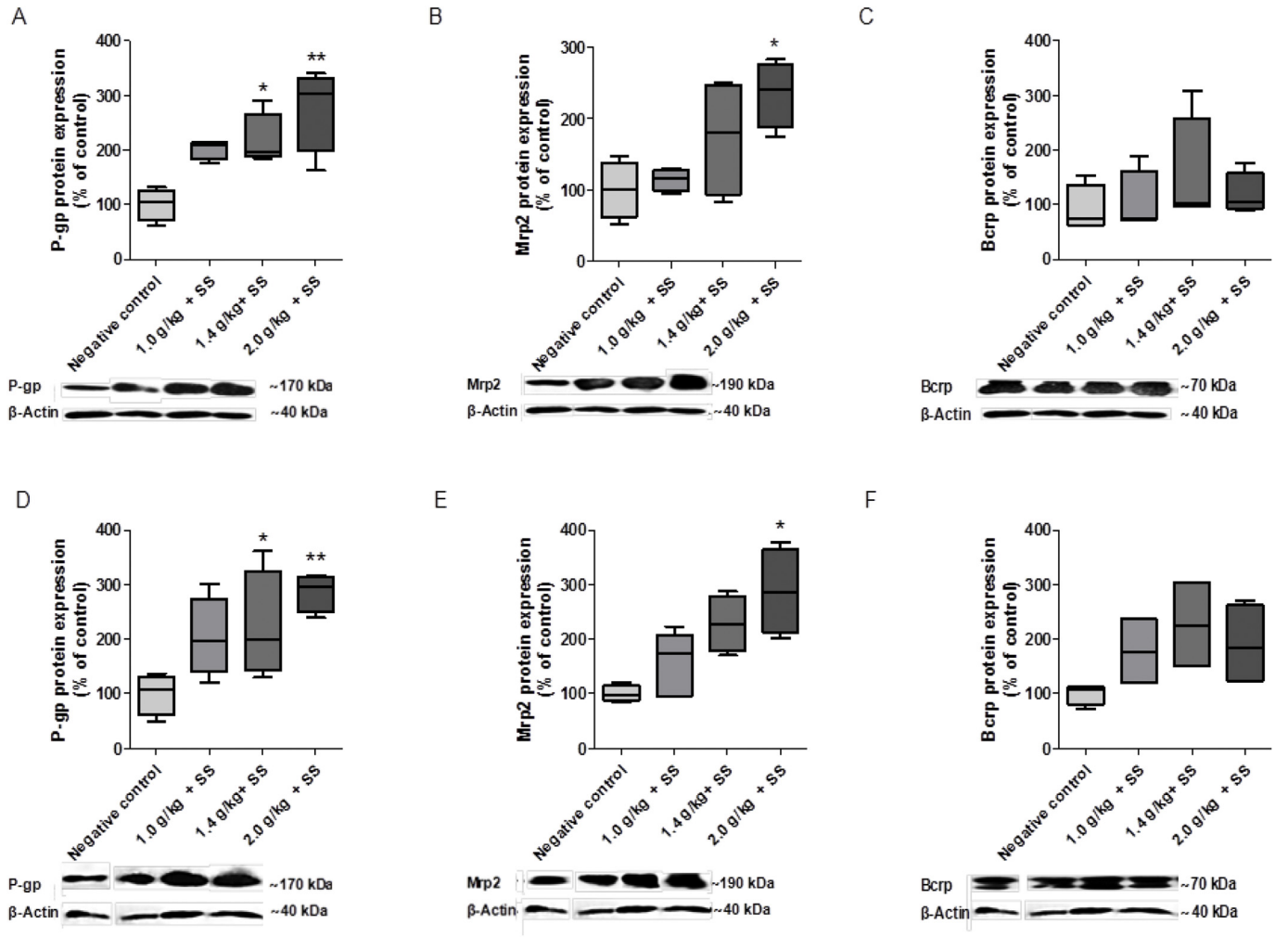

Fig. 3. Effect of *E. vesicaria* juice on hepatic ABC transporter expression. Representative immunoblot of P-gp (A, D), Mrp2 (B, E), and Bcrp (C, F) in liver of adult male (A,B,C) or female (D, E, F) mice. Animals were treated with saline solution (white bars) or 1 g/kg (light gray bars), 1.4 g/kg (medium gray bars) or 2 g/kg (dark gray bars) of *E. vesicaria* juice. Densitometric units were calculated as ratio between optical density of transporter and the load control band. Bars represent protein levels as percentages of the average value from control group. Results are expressed as median and the lower and upper quartiles of four mice per group. * = $p < 0.05$ and ** = $p < 0.01$ versus negative control, respectively (Kruskall Wallis and Dunn's post-hoc test).

used as a positive control in *in vivo* genotoxicity assessments (Krishna et al., 1995; Organization for Economic Cooperation and Development (OECD), 2016). For that purpose, we treated three groups of mice with an i.p. injection of 50 mg/kg of CP after chronic administration of the three doses of the vegetable juice ("protective effect" groups). The comet assay revealed a significant decrease in DI for the three doses, showing a significant inverse correlation between damage and juice dose (Pearson's $R = -0.613$, $p < 0.001$). For a deeper characterization of this effect, a Damage Reduction Index was calculated for each dose (refer to section 2.5) that confirmed a reduction of nearly eighty percent of the damage for the highest juice dose.

Moreover, the significant decrease in the frequency of Mn in PCE for

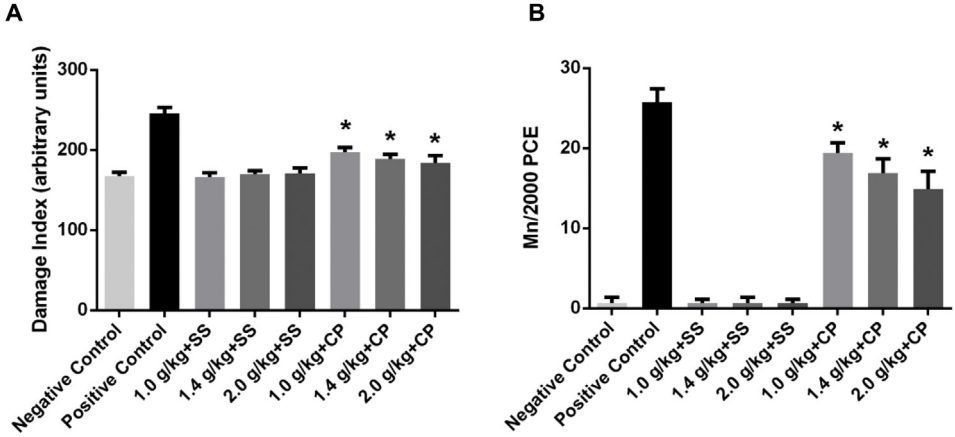

Fig. 4. Genotoxicity biomarkers. A: DNA damage in mice blood cells.Bars represent mean ± SD of the damage index in arbitrary units obtained through the alkaline comet assay (8 animals per group); *$p < 0.001$ versus positive control (Kruskal-Wallis with Dunn's post-hoc test). B: Frequency of micronucleated polychromatic erythrocytes (PCE) of mice bone marrow. Bars represent mean ± SD of the number of micronuclei in2000 PCE (8 animals per group); *$p < 0.05$ versus positive control (one-way ANOVA with Dunnett's post-hoc test).

Table 2
Cytotoxicity Indices and frequencies of Mn in NCE.

Treatment	CI (X ± SD)	Mn/2000 NCE (X ± SD)
Negative Control	0.52 ± 0.06	0.75 ± 0.71
Positive Control	0.50 ± 0.08	1.00 ± 0.76
1 g/kg b.w. + SS	0.55 ± 0.05	0.38 ± 0.74
1.4 g/kg b.w. + SS	0.54 ± 0.03	0.38 ± 0.74
2 g/kg b.w. + SS	0.48 ± 0.06	0.63 ± 0.52
1 g/kg b.w. + CP	0.55 ± 0.06	1.13 ± 1.13
1.4 g/kg b.w. + CP	0.53 ± 0.06	1.13 ± 0.35
2 g/kg b.w. + CP	0.47 ± 0.06	0.63 ± 0.74

SS: saline solution, CP: cyclophosphamide 50 mg/kg body weight.

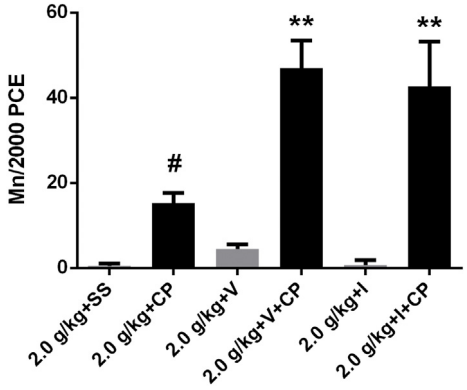

Fig. 5. Effect of hepatic ABC transporter inhibition on *E. vesicaria* juice antigenotoxicity. Frequency of micronucleated polychromatic erythrocytes (PCE) of mice bone marrow. Female mice were treated with 2.0 g/kg body weight of *E. vesicaria* juice. On day 15, animals were divided into six groups receiving an i.p. injection of saline solution (SS) or 50 mg/kg cyclophosphamide (CP) alone or in addition to an i.p. injection of either the P-gp inhibitor verapamil (50 mg/kg; V) or the Mrp2 inhibitor indomethacin (10 mg/kg; I). Two groups of animals were treated with verapamil or indomethacin alone for evaluation of basal toxicity of the ABC inhibitors. Bars represent mean ± SD of the number of micronuclei every 2000 PCE (4 animals per group); #p < 0.05 versus SS; **p < 0.001 versus CP (one-way ANOVA with Dunnett's post-hoc test).

the three juice doses *of E. vesicaria* suggests that this cruciferous also exerted a protective effect against clastogenic damage induced by CP metabolites. Furthermore, the significant correlation between this effect and juice dose (Spearman's R = −0.810, p < 0.001) allowed us to conclude that the protective effect of *E. vesicaria* was dose-dependent. In addition, Damage Reduction Indices for the Mn test were lower than for the comet assay but exceeded forty percent for the highest dose.

Since the health promoting effects of cruciferous vegetables has been vastly attributed to glucosinolate content, we requested collaboration from Dr. Niels Agerbirk (Department of Plant and Environmental Sciences, University of Copenhagen, Denmark) who performed LC-MS for the detection of glucosinolates on our *E. vesicaria* leaves. The main glucosinolates found in our accessions were glucoraphanin, glucoerucin, methoxyglucobrassicin and diglucothiobeinin (Bell et al., 2015; Lelario et al., 2012). However, these phytochemicals were present in extremely low concentrations below the limits of quantification of the method. Similar results were found in commercial varieties of *E. vesicaria* showing enhanced flavonol concentrations but extremely low concentration of glucosinolates (Bell et al., 2015).

Nonetheless, we could identify and quantify other types of phytochemicals in our plant, these being flavonoids, phenols and hydroxycinnamic acids. Given the fact that they were present in much higher concentrations than glucosinolates (Table 1), we propose that these phytochemicals could be responsible for the antigenotoxic effects that we observed.

Regarding the expression of ABC transporters, the significantly higher protein abundance of P-gp and Mrp2 in livers isolated from females compared to males in our experiments agrees with previous evidence reported in rodents proposing that these two transporters are regulated by sex hormones (Lu and Klaassen, 2007; Suzuki et al., 2006).

In turn, expression levels of hepatic ABC transporters remained unchanged after 24 h of a single i.p. dose of 50 mg/kg CP (Fig. 2). On the other hand, a significant induction of the protein expression of P-gp and Mrp2 after chronic oral administration of *E. vesicaria* juice was observed in both sexes (Fig. 3). This modulating effect of *E. vesicaria* could be attributed to flavonoids, as kaempferol interacts with Mrp2 and Bcrp transporters *in vivo* (Zheng et al., 2016). It is of relevance that the quali-quantitative phytochemical composition, and consequently their actions on the organisms, differ between cruciferous species. In this sense, *Diplotaxis tenuifolia* (wild rocket) shows a different pattern of modulation of hepatic ABC transporters (López Nigro et al., 2018) in contrast with *E. vesicaria* (present results). Exposure to *Diplotaxis tenuifolia* do not induce hepatic expression of P-gp, but expression of Mrp2 is decreased in females and increased in males showing sex-related differences; regarding Bcrp, its expression is strongly induced in

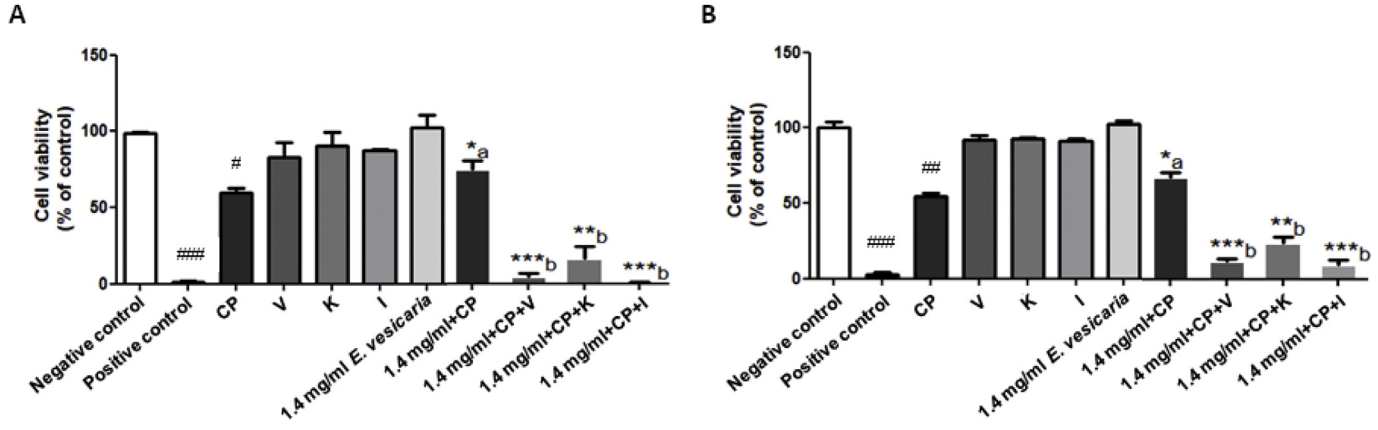

Fig. 6. Cytotoxicity assays in human liver cells. Bars represent percentage of viable HepG2/C3A cells respect to the negative control evaluated by MTT assay (Fig. 6A) or the Trypan Blue exclusion assay (Fig. 6B). Negative Control: saline solution; Positive control: 80 μM doxorubicin for 24 h; CP: 10 mM CP for 24 h; V: 35 μM verapamil for 24 h; K: 10 μM KO-143 for 24 h; I: 80 μM indomethacin for 24 h; 1.4 mg/ml: pretreatment with 1.4 mg/ml *E. vesicaria* juice for 48 h. Data represent the mean ± SD of four independent experiments. #p < 0.05 versus Negative control; ##p < 0.01 versus negative Control; ###p < 0.001 versus Negative control; a = *p < 0.05 versus CP; b = **p < 0.01 versus 1.4 mg/ml + CP, ***p < 0.001 versus 1.4 mg/ml + CP (Kruskal-Wallis, post-hoc Dunnett's test for multiple comparisons, *t*-test and Mann-Whitney's *U* test).

both male and female mice. Concerning participation of glucosinolates, although being present in very low concentrations in our accessions, it could not be discarded for *E. vesicaria* since sulforaphane and erucine induce P-gp and Mrp2 in human hepatic carcinoma cells (Harris and Jeffery, 2008). The differential behavior of both species may lay within variations in their phytochemical quantitative composition, since *D. tenuifolia* contains significantly higher concentrations of total phenols and hydroxycinnamic acids (López Nigro et al., 2018 and Table 1 of this manuscript). In this sense, consistent variations in the phenolic compounds between *E. vesicaria* and *D. tenuifolia have been reported* (Bell et al., 2015). These differences in the modulation pattern of hepatic ABC transporters could account for the differences found in the antigenotoxic effect of both cruciferous vegetables. Accordingly, the damage reduction index (DRI) for both genotoxicity biomarkers is higher for wild rocket than for common rocket (López Nigro et al., 2018 and present results, respectively).

Moreover, since ABC transporters are able to expel CP and its metabolites to the cellular exterior (Brayboy et al., 2017, 2013; Joy et al., 2012), the fact that repeated exposure to *E. vesicaria* positively modulated the ABC transporters in liver could contribute to reduce CP-induced genotoxicity. Regarding Mrp2, it regulates the rate of biliary excretion of GSCP (metabolite of 4OH-CP). Although this metabolite is inactive, its higher accumulation in Mrp2-deficient rats facilitates the oxidative metabolic pathway of 4OH-CP with the consequent increase in cytotoxicity (Qiu et al., 2003). In addition, a polymorphic variant of P-gp, which correlates with altered expression and activity of the transporter, reduces the elimination rate of CP and 4OH-CP in patients with glomerulonephritis (Joy et al., 2012), thus indicating that P-gp is also involved in the pharmacokinetics of CP and its metabolites. In support of our hypothesis that ABC transporters participate in the antigenotoxic action of *E. vesicaria*, the present findings show that the protective effect of the cruciferous against CP-induced genotoxicity is reduced by P-gp (50 mg/kg verapamil) as well as Mrp2 (10 mg/kg indomethacin) inhibition (Fig. 5). On the light of this evidence, we propose that induction of hepatic Mrp2 and P-gp after chronic intake of *E. vesicaria* juice could facilitate biliary excretion of CP or its metabolites, thus interfering with its genotoxicity in blood and bone marrow cells.

To gain further knowledge on whether hepatic ABC transporters are involved in the effect of *E. vesicaria* on CP-induced toxicity, we designed *in vitro* experiments in human hepatoma cells (HepG2/C3A). This cell line is a sub clone derived from HepG2 used as model for toxicological studies since it displays specialized functions of the normal liver such as xenobiotic metabolism and ABC transporter pathways (Belkahla et al., 2018; Hewitt and Hewitt, 2004). Cytotoxic concentrations of CP (10 mM) were evaluated by MTT and Trypan Blue assays, which evidence mitochondrial activity and cell membrane integrity, respectively. Under these experimental conditions, 1.4 mg/ml *E. vesicaria* juice attenuated CP-induced cytotoxicity on HepG2/C3A cells, but this effect was no longer evident when P-gp (35 μM verapamil) or Mrp2 (80 μM indomethacin) were inhibited. Interestingly, in the human hepatoma cell line, BCRP was also involved in the protective effect of *E. vesicaria*. The discrepancies with *in vivo* findings may be due to the fact that not all orally administered compounds reach enough plasma concentrations to exert their effect and, in addition, there are no equivalences between the doses used *in vivo* with respect to the concentrations used in *in vitro* tests. However, possible differences between species cannot be ruled out.

Present findings suggest that efflux of CP and/or its metabolites through ABC transporters may diminish their toxicity, and an increase in their expression of these efflux pumps could be one of the mechanisms through which *E. vesicaria* exerts its protective effect. Furthermore, they add evidence to that already reported by Lamy, who observed that phase II metabolic enzymes are involved in the protective effect of the species *Eruca sativa* against benzo(a)pyrene-induced genotoxicity (Lamy et al., 2008). Hepatic phase II metabolic enzymes and ABC transporters act in a coordinated way to detoxify the organism. It is possible that phytochemicals present in *Eruca* spp. act synergistically by modulation of both systems, generating an antigenotoxic effect at doses comparable to the average intake in humans. Repeated rocket intake would facilitate the homeostatic response of the body in its protection against environmental insults such as those elicited by oral exposure to xenobiotics.

Further *in silico* and *in vitro* experiments conducting to identify the secondary metabolites involved in the modulation of ABC transporters in each species are the goal of our future investigations.

5. Conclusions

It appears that incorporation of fresh leaves of *E. vesicaria* to the diet could induce the expression of hepatic ABC transporters contributing to reduce the risks of cellular burden of toxic compound exposure. These results give further support to the beneficial effect that was previously observed *in vitro* and in non-vertebrate species as *Drosophyla melanoganster* (Barillari et al., 2005; Villatoro-Pulido et al., 2012).

On the other hand, it should be taken into account that rocket consumption could lead to drug-diet interactions that might negatively affect the treatment with agents that induce DNA damage, and with drugs whose pharmacokinetics depend on the action of ABC transporters.

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Declaration of competing interest

The authors declare that they have no known financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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