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ORIGINAL PAPER

Persistence of antioxidant, antimutagenic, and antigenotoxic properties of *Polygonum maritimum* L. extract after in vitro digestion supports its high potential as a functional food ingredient

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Abstract

Continuous exposure to xenobiotic-contaminated food causes adverse effects that may lead to health complications, including cancer. Halophytes are rich in polyphenols that exhibit notable biological properties and may contribute to cancer prevention. However, digestion can modify the chemical structure of polyphenols, potentially reducing their biological properties. This study investigated the protective effects of a polyphenol-rich extract from the halophyte *Polygonum maritimum* L. (PME) against contaminants-induced toxicity and assessed the persistence of these properties following in vitro digestion. LC-DAD-ESI/MS^n analysis revealed that the phenolic composition of PME decreased considerably after digestion. Nevertheless, the antioxidant activity of PME, measured as a decrease in reactive oxygen species levels in H_2O_2-challenged HepG2 cells, persisted post-digestion. The extract showed enhanced anti-inflammatory activity after digestion, as shown by the ability to reduce $^{\cdot}NO$ production in lipopolysaccharide-stimulated RAW 264.7 cells. The antigenotoxicity of PME against the contaminant benzo[a]pyrene (BaP) in Caco-2 and HepG2 cells remained significant following upper gastrointestinal tract digestion (DPME), whereas the antigenotoxicity against H_2O_2 only persisted in Caco-2 cells. After colonic fermentation (FPME), antigenotoxicity was observed against H_2O_2 in HepG2 cells. PME also displayed antimutagenicity towards BaP in *Salmonella typhimurium* TA98 and TA100 strains, which remained relevant in DPME, but was drastically reduced in FPME. Furthermore, *Danio rerio* fed with PME and BaP showed reduced negative effects in terms of size and lipid peroxidation. These results suggest that PME can protect against contaminants-induced toxicity along the gastrointestinal tract, maintaining bioactivity until colonic fermentation. Thus, PME can be a promising functional food ingredient for health improvement.

Keywords Antioxidant · Antimutagenic · Antigenotoxic · Phenolics · Sea knotgrass · Simulated digestion

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Introduction

Consumption of vegetables and fruits rich in polyphenols has been associated with cancer prevention because of the antioxidant and anti-inflammatory properties of these compounds [1]. The capacity of polyphenols to protect DNA from damage and to promote DNA damage repair may also play a role in cancer prevention [2, 3]. The protective effects of polyphenols against cancer initiation may include mechanisms such as direct scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS), induction of antioxidant defenses (e.g., glutathione, and enzymes like catalase and superoxide dismutase) and the modulation of phase I [e.g., cytochrome P450 1A1 (CYP1A1)] and phase II (e.g., glutathione *S*-transferase) enzymes involved in the metabolization and detoxification of xenobiotics, respectively [4]. Besides the conventional dietary sources of polyphenols, medicinal plants such as salt tolerant plants (halophytes) are rich in phenolic compounds and with high antioxidant activity [5], among other bioactivities of interest, and represent a noteworthy source of chemopreventive compounds to include in the human diet. *Polygonum maritimum* L. (sea knotgrass) is a halophyte found on sandy coasts of several geographical locations, including the Mediterranean region [6]. Extracts from the aerial parts of *P. maritimum* collected in Southern Portugal are rich in phenolic compounds, predominantly flavonoids [7–9]. These extracts have notable biological activities, including antioxidant, antigenotoxic, anti-inflammatory, anti-diabetic, and neuroprotective properties, as reported in various studies [7–10]. Therefore, *P. maritimum* is a promising source of compounds for preventing degenerative diseases.

Polyphenols occur in plants mostly in glycosylated forms [11], which is the most common form of human consumption in the diet. Depending on the structural complexity and degree of polymerization [11], polyphenols undergo several transformations during digestion before they become accessible for absorption (bioaccessibility) to exert effects on the target tissue. During digestion, the activity of enzymes and acidic and mild-alkaline conditions in the stomach and small intestine, respectively, convert polyphenols in low-molecular-weight derivatives that can be absorbed by intestinal cells and further metabolized in the liver [12]. Notably, only a small portion (5–10%) of the total phenolic intake is absorbed in the small intestine, while the rest passes directly to the colon to be metabolized by colonic microbiota [12–14]. Some of these metabolites can be absorbed in the colon, where they induce their beneficial effects locally, or be metabolized in the liver and distributed to other tissues [15]. Chemical modification of polyphenols during digestion can affect bioaccessibility, bioavailability, and biological activities, which highlights the importance of investigating the effects caused by digested compounds and not solely the original ones. Foods, beverages, and extracts of plants rich in polyphenols have been subjected to in vitro digestion [16–21] to investigate the stability of phenolic compounds [22] and structure-related bioactivities after digestion.

Toxic chemicals can contaminate food and drinking water, posing a significant global food safety concern. Many of these compounds are associated with severe adverse health effects, including metabolic and reproductive disorders, intestinal inflammation that compromises the intestinal barrier, and an increased risk of cancer [23–25]. Benzo[a]pyrene (BaP), a known carcinogen to humans (group 1, IARC classification) [26], is a toxic chemical found in foods, particularly in grilled meat and fish under charcoal and wood fires, as it can be formed as a neo-formed contaminant during food cooking and processing at high temperatures [27]. BaP stimulates its bioactivation by inducing the expression and activity of phase I-metabolizing enzymes *via* aryl hydrocarbon receptor (AHR). CYP1A1 is one of the most important hepatic and intestinal enzymes involved in this process that ultimately generates reactive metabolites with genotoxic and carcinogenic potential able to covalently bind to DNA and form adducts [28]. In addition, ROS can be formed during the process of metabolic activation of BaP, possibly causing oxidative stress and damaging cellular constituents, including DNA. Increased risk of carcinogenicity can arise from failure to correctly repair DNA damage, leading to mutations, and repeated exposure to toxic chemicals that cause persistent inflammation in the gastrointestinal tract, increasing the levels of inflammatory mediators, such as ROS, RNS and cytokines [29]. Oral administration of BaP has been shown to induce oxidative stress and inflammatory biomarkers in the colon of BALB/c mice and the deregulation of Wnt/β-catenin signaling pathway involved in the development of colorectal cancer [30]. Additionally, BaP induced DNA damage in the liver and colon of Apc^{Min} mice [31]. Considering the inevitable dietary exposure to toxic chemicals like BaP, the main purpose of this study was to investigate the potential application of the *P. maritimum* extract in food and nutraceutical industries for health purposes by elucidating its protective effects towards BaP-related toxicity and predict the impact of digestion on the stability of the chemical composition and protective effects of the extract.

Materials and methods

Plant material and extraction

Aerial organs (leaves and shoots) of *P. maritimum* were collected in Southern Portugal (Faro beach) in August 2018.

The species was identified by Dr. Luísa Custódio (Centre of Marine Sciences, University of Algarve, Portugal) and a voucher specimen was maintained in the XtremeBio laboratory (voucher nº MBH22). Biomass was cleaned, oven dried for five days at 40 °C, converted into powder and stored at −20 °C. The *P. maritimum* aerial part ethanol extract (PME) was prepared by stirring dried plant material and absolute ethanol in the dark (1:10, *w/v*) at 100 rpm and 25 °C for 24 h. The extract was filtered (Whatman No 04 paper and syringe filter 0.2 µm), and the solvent was evaporated in a Rotavapor under low pressure at 40 °C and then with gaseous nitrogen. The dried extract was stored at −20 °C.

In vitro simulation of human digestion

The upper gastrointestinal tract digestion

The upper gastrointestinal tract digestion of PME was in vitro simulated using a methodology developed by Gil-Izquierdo et al. [32] adapted as described by Oliveira et al. [19]. In brief, for gastric digestion, pepsin (18.2 mg/mL) was added to a sample of PME (1 mg/mL), followed by acidification to pH 2.0 using HCl and subsequent incubation in shaking water bath at 100 rpm and 37 °C for 2 h. For the small intestine digestion, pancreatin (4 mg/mL; enzymatic components including trypsin, amylase and lipase, ribonuclease, and protease) and bile salts (25 mg/mL) were added to the post-gastric sample, which was put in contact with a dialysis tubing (molecular mass cut off 12 kDa) containing the volume of $NaHCO_3$ solution required to adjust the pH of the sample to 7.5 and subsequently incubated in the conditions indicated above. The sample was centrifuged, lyophilized, and stored at −20 °C. The final product of upper gastrointestinal tract digestion represents the digested extract that becomes available in the colon (DPME). A control sample, without the extract, was carried out simultaneously in the simulation of digestion.

Colonic fermentation

In vitro colonic fermentation of DPME was performed using methodology adapted from Correa et al. [16]. Briefly, a carbonate-phosphate buffer (pH 7.0) containing a solution of macro (9.240 mg/mL $NaHCO_3$, 3.542 mg/mL $Na_2HPO_4{\cdot}2H_2O$, 0.470 mg/mL NaCl, 0.450 mg/mL KCl, 0.227 mg/mL $Na_2SO_4{\cdot}10H_2O$, 0.055 mg/mL $CaCl_2$ - anhydrous, 0.100 mg/mL $MgCl_2{\cdot}6H_2O$, 0.400 mg/mL urea) and micro (36.80 mg/L $FeSO_4{\cdot}7H_2O$, 11.59 mg/L $MnSO_4{\cdot}H_2O$, 4.40 mg/L $ZnSO_4{\cdot}7H_2O$, 1.20 mg/L $CoCl_2{\cdot}6H_2O$, 0.98 mg/L $CuSO_4{\cdot}5H_2O$, 0.174 mg/L $Mo_7(NH_4)_6O_{24}{\cdot}4H_2O$) elements and 0.8% (*w/v*) glucose was used as the basal culture medium for the colonic fermentation. DPME was added to the medium (1:20, *w/v*), and this mixture was purged with nitrogen gas until the anaerobic indicator (methylene blue) turned colorless. Fresh fecal samples were collected from three healthy human donors (25–30 years), who had not used antibiotics within the previous three months and had followed a diet low in antioxidants for at least three days prior to sampling. Each fecal sample was processed individually under anaerobic conditions, homogenized in anaerobic buffer, and subsequently combined in equal proportions to obtain a pooled fecal inoculum. This pooling strategy was adopted to reduce interindividual variability and to obtain a representative average microbial community, as commonly recommended in in vitro colonic fermentation studies [33]. The pooled fecal inoculum was added to the fermentation medium (1:20, *w/v*) in a vessel previously purged with nitrogen gas and hermetically sealed. Fermentation was conducted under continuous stirring (60 rpm), kept in the dark, at 37 °C for 24 h. After the fermentation, the sample was centrifuged, filtered and lyophilized. The resulting material represents the fraction that reached the colon and was metabolized by colonic microbiota (FPME).

Chemical composition

Total phenolics content

The total phenolic content was determined using the Folin-Ciocalteu method [34] adapted for microplate assay. In brief, 10 µL of the samples (PME, DPME or FPME) were mixed with 50 µL of Folin-Ciocalteu reagent (1:10 *v/v* in deionized water) and 40 µL of 75 mg/mL Na_2CO_3. This mixture was incubated for 1 h at room temperature in the dark and the absorbance was measured at 760 nm wavelength. Deionized water replaced Folin-Ciocalteu reagent and Na_2CO_3, and absolute ethanol replaced the samples in the blank and control, respectively. The absorbance of the samples (absorbance = absorbance sample – absorbance blank – absorbance control) was correlated with a gallic acid standard curve to obtain total phenolic content values expressed in µg gallic acid equivalents (GAE)/mL. This correlation was used to express the concentration of samples in GAE in bioactivity assays.

Analysis of phenolic composition

The chemical composition was determined by liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry (LC-DAD-ESI/MS^n), as previously described [7, 35]. The samples were dissolved in a methanol: water (80:20, *v/v*) mixture (5 mg/mL) and filtered through a 0.22 µm membrane prior to analysis. The phenolic profile was determined by liquid chromatography

coupled to diode array detection and electrospray ionization tandem mass spectrometry (LC-DAD-ESI/MS^n) using a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a quaternary pump, autosampler maintained at 5 °C, degasser, and thermostatted column compartment, exactly as previously described [7]. Chromatographic separation was carried out on a C18 reversed-phase column (3 µm, 4.6 × 150 mm) maintained at 35 °C, using water with 0.1% formic acid (solvent A) and acetonitrile (solvent B) as mobile phases, at a flow rate of 0.5 mL/min. The gradient elution consisted of an initial isocratic step followed by a gradual increase in organic solvent and column re-equilibration. UV–Vis spectra were recorded at 280 and 370 nm wavelengths. Mass spectrometric detection was performed in negative ion mode on a linear ion trap mass spectrometer equipped with an ESI source, operating over an *m/z* range of 100–1500. Phenolic compounds were identified based on retention time, UV–Vis characteristics, and mass fragmentation patterns, by comparison with reference standards or literature data. Quantification was carried out using calibration curves of available standards or structurally related compounds, and results were expressed as mg/g of extract.

Cell models, culture conditions, and cytotoxicity assays

Cultures of *Salmonella typhimurium* bacterial strains TA98 and TA100 carrying frameshift and base-pair substitution mutations, respectively, were grown in liquid medium (Oxoid nutrient broth supplemented with 28 µg/mL ampicillin) at 37 °C and 120 rpm and the optical density was monitored at 595 nm wavelength to measure bacterial proliferation. Human hepatoma cell line HepG2 and colon carcinoma cell line Caco-2 were obtained from the European Collection of Cell Cultures (UK) and were cultured as a monolayer in Minimum Essential Medium (MEM; Gibco by Life Technologies) and high glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco by Life Technologies), respectively, supplemented with 2 mM L-glutamine (Gibco by Life Technologies), 1% (*v/v*) non-essential amino acids, and 10% (*v/v*) heat-inactivated fetal bovine serum (FBS; Dominique Dutscher) at 37 °C in a humidified incubator with 5% CO_2 [36, 37]. In addition, the culture medium of Caco-2 cell line was supplemented with 1% (*v/v*) penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively; Gibco by Life Technologies). The culture medium was replaced every 2 days. Cell cultures were split every week by trypsinization and HepG2 and Caco-2 cells were seeded at 2.0×10^6 cells/75 cm^2 flask and 1.0×10^5 cells/75 cm^2 flask, respectively. Cells were used until 10 passages after thawing. The media mentioned above were used for the preparation of test treatments for the experiments, only replacing 10% (*v/v*) FBS with 0.5% (*v/v*) FBS. The RAW 264.7 mouse macrophage cell line was obtained from DMSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and cultured in DMEM supplemented with 10% (*v/v*) FBS, 2 mM L-glutamine and penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively) at 37 °C and 5% CO_2 in a humidified incubator.

The cytotoxicity of PME, DPME or FPME was assessed in RAW 264.7 cells using the sulforhodamine B (SRB) colorimetric method [37]. The effect of PME, DPME or FPME and treatments with BaP (purity ≥ 96%; Sigma-Aldrich) or H_2O_2 (Sigma-Aldrich) on the viability of HepG2 and Caco-2 cells was assessed to ensure suitable working conditions, using the PrestoBlue high sensitivity cell viability reagent (Invitrogen, Thermo Fisher) according to the manufacturer's instructions [38].

Antioxidant activity

Antioxidant activity was assessed using the redox probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma-Aldrich) in HepG2 cells [39]. For the experiment, cells were seeded at a density of 5×10^4 cells/well in 96 well-plates at 37 °C and 5% CO_2 for 24 h. The culture medium was removed, cells were washed with phosphate buffered saline without calcium and magnesium (PBS, Gibco by Life Technologies), and MEM (without phenol red) containing 50 µM H_2DCFDA was added to the cells for 1 h incubation in the dark at 37 °C and 5% CO_2. The medium was removed, cells were washed, and the test treatments were added. Five test concentrations of PME (0.313–5 µg GAE/mL), DPME (0.156–2.5 µg GAE/mL) or FPME (0.078–1.25 µg GAE/mL) were added to the controls of sample, to infer about their effects, and to the pre-treatment groups. In addition, the solvents of the samples [0.1% (*v/v*) absolute ethanol or 2.5% (*v/v*) PBS] were added to the respective negative and positive controls. Subsequently, cells were incubated at 37 °C and 5% CO_2 for 20 h. Cells in the positive control and pre-treatment groups were treated with 1 mM H_2O_2 for 20 min in the same incubation conditions. The medium was removed, and PBS was added for subsequent measurement of ROS levels using a fluorescence plate reader at 485 nm (excitation) and 530 nm (emission) wavelengths. Data were normalized to the positive control (1 mM H_2O_2), which was considered as 100% of intracellular ROS levels.

Anti-inflammatory activity

Anti-inflammatory potential was assessed using the Griess reaction in RAW 264.7 cells [40]. Cells detached by scraping, with a proportion of dead cells below 5% according

to the Trypan blue exclusion test, were plated at a density of 1.5×10^5 cells/well and incubated at 37 °C and 5% CO_2 for 24 h. Cells were treated with different concentrations of PME, DPME or FPME (0.94–60 µg GAE/mL) for 1 h, followed by stimulation with lipopolysaccharide (LPS) solution (Sigma-Aldrich) and subsequent incubation for 24 h. Similar treatments, except for the presence of LPS, represented the negative control and 50 µM dexamethasone was used as the positive control of the experiment. The absorbance was measured at 540 nm wavelength and the ˙NO produced was estimated using the Griess reagent system kit (Promega) and a nitrite calibration curve according to the manufacturer's instructions. The results were calculated through the graphical representation of the percentage of inhibition of ˙NO production versus the sample concentration and were expressed as the concentration of sample that causes 50% inhibition of ˙NO production (EC_{50}).

Antimutagenicity

Antimutagenicity was evaluated under metabolic activation [S9 mix: 10% (*v/v*) S9 fraction obtained from the liver of Sprague-Dawley rats induced with phenobarbital/5,6-benzoflavone (TrinovaBiochem); and the co-factors 8 mM magnesium chloride, 33 mM potassium chloride, 4 mM nicotinamide adenine dinucleotide phosphate and 5 mM glucose-6-phosphate prepared in phosphate buffer (pH 7.4; Sigma-Aldrich)], using the histidine auxotroph *S. typhimurium* strains TA98 and TA100 in the Ames test [41]. PME was tested using the traditional method of plate incorporation (co-incubation) and DPME and FPME were tested using a miniaturized version of the method, due to the limited sample available, in collaboration with GenEvolutioN. For the traditional method, the following constituents were added to a test tube: 2 mL of top agar (10 mL of 0.5 mM histidine/biotin solution per 100 mL agar), 50 µL of test solution, 500 µL of S9 mix and 100 µL of *S. typhimurium* culture in liquid medium grown for 14–15 h. The test solutions were added as follows: negative control, dimethyl sulfoxide (DMSO; solvent of BaP) and absolute ethanol (solvent of sample); positive control, 3 µg BaP/plate and absolute ethanol; control of sample, 3.2, 6.4 or 12.8 µg PME/plate; co-treatment, 3.2, 6.4 or 12.8 µg PME/plate and 3 µg BaP/plate. The mixtures were poured onto minimal glucose agar plates and incubated at 37 °C for 48 h. For the miniaturized version of the Ames test, 1.2 µL of test solutions were added to wells of a 96-well microplate (one well per condition), followed by the addition of 6 µL of solution A, 15 µL of bacteria, and 150 µL of solution B. The test solutions were added as follows: negative control, DMSO and PBS (solvent of samples); positive controls, 10 or 60 ng BaP/well (TA98 or TA100 strains, respectively) and PBS; co-treatment, 10 or 60 ng BaP/well (TA98 or TA100 strains, respectively) and 25, 50 or 100 ng DPME/well or 6.25, 12.5 or 25 ng FPME/well. All samples (PME, DPME or FPME) were also tested alone at the indicated concentrations for control purposes. Empty wells of the 96-well microplate were filled with 150 µL of solution B. Plates were sealed with parafilm film and incubated overnight at 37 °C under shaking. After the pre-incubation period, the following constituents were added to a test tube: 3 mL of supercooled overlay agar, 720 µL of solution C, and the content of one well from the 96-well microplate after homogenization. Then, 650 µL of the mixture were dispensed in 5 wells of a 25-well plate containing 2 mL of minimal glucose agar. After agar solidification, the plates were turned over and incubated at 37 °C for 72 h. The revertant colonies were counted and the antimutagenicity was expressed as percentage of inhibition of mutagenic effect, calculated as: 100 − [(number of revertant colonies treated with sample and mutagen/number of revertant colonies treated with the mutagen) x 100] [42]. The traditional and miniaturized versions of the Ames test were carried out in triplicate or in three independent experiments, respectively. Cytotoxicity was judged when reduction in the number of revertant colonies, clearing or diminution of the bacterial background lawn, and presence of microcolonies were observed. The composition of solutions A, B and C are under protection of commercial rights.

Antigenotoxicity

The antigenotoxicity was investigated using the comet assay (alkaline single-cell gel electrophoresis) [43]. HepG2 and Caco-2 cells were seeded in 96 well-plates at a density of 5×10^4 and 1×10^4 cells/well, respectively, and incubated at 37 °C and 5% CO_2 for 24 h. The culture medium was removed, and cells were exposed to the treatments. Treatments for evaluation of antigenotoxicity against H_2O_2 were applied as follows: 0.1% (*v/v*) absolute ethanol or 2.5% (*v/v*) PBS were added to the respective negative and positive controls and three concentrations of PME (1.25, 2.5 or 5 µg GAE/mL), DPME (0.625, 1.25 or 2.5 µg GAE/mL) or FPME (0.313, 0.625 or 1.25 µg GAE/mL) were added to the controls of sample and to the pre-treatment groups. The treated cultures were incubated at 37 °C and 5% CO_2 for 20 h and 200 µM or 100 µM H_2O_2 were added to the positive control and to the pre-treatment groups in HepG2 and Caco-2 cells, respectively, and subsequently incubated under the previously mentioned conditions for 30 min. Treatments for assessment of antigenotoxicity against BaP were applied as follows: 0.25% (*v/v*) DMSO and 0.1% (*v/v*) absolute ethanol or 2.5% (*v/v*) PBS were added to the negative control; 50 µM BaP and 0.1% (*v/v*) absolute ethanol or 2.5% (*v/v*) PBS were added to the respective positive

controls; and the above mentioned concentrations of PME, DPME or FPME were added to the controls of sample and to the co-treatment groups with 50 µM BaP. The treated cultures were incubated under the conditions mentioned above for 20 h. The medium was removed, the cells were washed with PBS, trypsinized, and subsequently collected in medium and centrifuged (100 *g*, 5 min, 4 °C). Cells were resuspended in PBS (4 °C), homogenized, and divided in two aliquots to generate duplicates of each sample. Each sample was mixed with 50 µL 0.5% (*w/v*) low melting point agarose (Sigma-Aldrich) at 37 °C and a drop was placed on glass slides previously coated with 1% (*w/v*) normal melting point agarose (Sigma-Aldrich) and subsequently covered with a coverslip. After gel solidification, coverslips were removed, and the slides were immersed in lysis buffer (1% *w/v* sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% *v/v* DMSO, 1% *v/v* Triton X-100, pH 10; Sigma-Aldrich) protected from the light for 1 h. Electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13; Sigma-Aldrich) covered the slides for 40 min, followed by electrophoresis at 25 V for 20 min. A neutralization buffer (0.4 M Tris, pH 7.5; Sigma-Aldrich) was used to wash the slides for 5 min (three times), and these were immersed in cold ethanol and left to dry overnight in the dark. The results were visualized using fluorescence microscopy by staining the gels with propidium iodide. One hundred cells per treatment were scored in accordance with five classes, from low (0) to high DNA damage (4). The results obtained were normalized to the respective positive controls, which were considered as 100% comet score.

Relative expression of *CYP1A1*

Cells treatment

The relative expression of *CYP1A1* was assessed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). HepG2 and Caco-2 cells were seeded in 6-well plates at 8.6×10^5 cells/well and 2×10^5 cells/well, respectively, and incubated at 37 °C and 5% CO_2 for 24 h. The culture medium was discarded, and cells were treated with only 1 µM BaP (positive control) or co-treated with 1 µM BaP and PME (1.25, 2.5 or 5 µg GAE/mL) or DPME (0.625, 1.25 or 2.5 µg GAE/mL) or FPME (0.313, 0.625 or 1.25 µg GAE/mL) for 20 h in the conditions previously mentioned. The concentrations of the samples indicated above were tested in parallel (controls of sample), as well as their solvents (0.1% *v/v* absolute ethanol or 2.5% *v/v* PBS) and the solvent of BaP (0.25% *v/v* DMSO) for comparison with untreated cells.

RNA extraction and cDNA synthesis

The medium was discarded, and cells were lysed with Tri Reagent (Molecular Research Center). For total RNA extraction, samples were mixed with 10% (*v/v*) chloroform and centrifuged at 1200 *g* and 4 °C for 15 min. The upper layer was recovered, and isopropanol was added to each sample (1:1, *v/v*), followed by incubation at room temperature for 15 min to allow RNA precipitation. Samples were centrifuged at 1200 *g* and 4 °C for 15 min. The supernatant was discarded, and 70% (*v/v*) ethanol was added, followed by centrifugation at 1000 *g* and 4 °C for 10 min (step performed twice). The total RNA was left to dry at room temperature for 10 min, followed by the addition of ultra-pure water and subsequent storage at −80 °C. Reverse transcription of 3 µg total RNA was executed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions to obtain cDNA.

qPCR analysis and normalization

Real time quantitative PCR was executed using reaction mixtures composed of 5 µL Taqman Universal PCR Master Mix and 0.5 µL Taqman Gene Expression Assay (Applied Biosystems, Thermo Fisher Scientific) and 1 µL cDNA (30 ng) for a final volume of 10 µL in a StepOnePlus Real Time PCR System (Applied Biosystems) under the indicated conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression was determined using the comparative threshold cycle (Ct; $2^{-\Delta\Delta Ct}$) method, normalizing the results of treated cells to those of untreated cells and using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as reference gene. Taqman gene expression assay references were Hs00153120_m1 (*CYP1A1*) and Hs99999905_m1 (*GAPDH*). For HepG2 cells, two independent total RNA extractions were performed, and two independent reverse transcriptions were carried out for one RNA extraction and one for the second RNA extraction. For Caco-2 cells, one experiment was performed.

Zebrafish assay

Experimental setup and diet preparation

Zebrafish (*Danio rerio*) was used for in vivo evaluation of protective effects of PME against BaP-induced toxicity. Diets were prepared by mixing commercial fish diet (SPAROS) with: (i) PME and BaP dissolved in absolute ethanol and acetone, respectively (co-treatment); (ii) BaP and absolute ethanol (positive control); (iii) PME and acetone

(control of sample); (iv) acetone and absolute ethanol (negative control). The solvents were evaporated, and the diets were reduced to powder (< 100 µm). Newly fertilized eggs (obtained as previously indicated [44]) were collected and kept at 26 °C under photoperiod of 14:10 h (light: dark; FOC 200i Cooled Incubator) until 6 days-post fertilization (dpf).

Exposure and sampling

Eighty larvae were placed in flasks containing 125 mL of dechlorinated water (4 flasks per treatment) and fed three times a day with the diets indicated above, testing the concentrations 2.5, 5 or 10 mg PME/g diet and 3.75 mg BaP/g diet from 6 dpf to 16 dpf under the conditions previously mentioned. The water was changed daily. At 16 dpf, larvae were grouped in pools ($n = 8$ pools/treatment, 20 larvae/pool), anesthetized and measured (40 larvae/treatment, 5 larvae/pool), and then sacrificed with an anesthetic overdose of 300 mg/L tricaine methanesulfonate (sodium bicarbonate was added to prevent acidification of the solution). Larvae were homogenized by an ultrasonic cell disruptor and centrifuged (4 °C, 10 000 *g*, 20 min), followed by recovery of the supernatant and respective protein quantification using the Bradford method [45] (standard: bovine serum albumin).

Ethical approval and compliance

Bioassay with zebrafish was performed in compliance with the European Directive 2010/63/EU and the Portuguese Decreto-Lei 113/2013, on the protection of animals used for scientific purposes. The project received approval for research ethics from the Animal Welfare and Ethics body (ORBEA – CIIMAR) and the bioassay was carried out in the facilities of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR, Matosinhos, Portugal).

Lipid peroxidation (LPO)

Lipid peroxidation was determined using the thiobarbituric acid assay based on the reaction between malondialdehyde (MDA) and thiobarbituric acid to determine MDA levels [46]. Sample (supernatant of a larval homogenate, 150 µL) was mixed with 16.8 µL trichloroacetic acid, centrifuged (4 °C, 5000 rpm, 20 min), and 120 µL of supernatant were added to 120 µL 0.1 M EDTA and 720 µL of a solution containing 1% (*w/v*) thiobarbituric acid, 0.05 M NaOH, and 0.025% (*w/v*) butylated hydroxytoluene. Blanks and controls were included in the experiment, replacing the sample by 120 µL water and 120 µL phosphate buffer, respectively. The reaction mixtures were boiled for 30 min in the dark, followed by absorbance measurement at 532 nm wavelength. These values were used to calculate the nmol of MDA equivalents per mg of protein.

Statistical analysis

Data are presented as the mean of three independent experiments with associated standard error of the mean (mean ± SEM), unless indicated otherwise. Data normality and homogeneity of variance were assessed with Shapiro-Wilks test and Brown-Forsythe test, respectively. One-way analysis of variance (ANOVA) test followed by Dunnett's multiple comparison test or Kruskal Wallis test followed by Dunn's multiple comparisons test were applied to analyze the differences between samples. Statistically significant differences were considered when $p < 0.05$, using the GraphPad Prism (version 8.2.1).

Results and discussion

Impact of in vitro simulated digestion on phenolic composition

Prediction of PME composition and respective fate during digestion was assessed by in vitro simulated upper gastrointestinal tract digestion and colonic fermentation, yielding the colon-available fraction (passed from the small intestine to the colon; DPME) and the post-colonic fermentation fraction (metabolized by colonic microbiota; FPME). The chemical profile of PME was previously described [7], revealing a phenolic composition rich in flavan-3-ols and flavonols, namely catechin and four β-type (epi)catechin oligomers (dimer and trimers), and one quercetin glycoside and two myricetin glycosides, respectively. The impact of digestion on the chemical composition of PME was investigated by LC-DAD-ESI/MS^n, showing that only catechin and two β-type (epi)catechin trimers were detected in DPME and no compounds were detected in FPME (Table 1). Considering that comparisons with previous analysis may be influenced by batch-to-batch variability, instrument performance drift and ionization efficiency, concentration differences were not considered; however, trends were found, indicating that β-type (epi)catechin trimers and catechin identified in DPME decreased in comparison to PME [7].

In line with our study, an aqueous infusion from the halophyte *Capparis spinosa* L. (flower tops and stalks) showed total loss of flavonol glycosides after in vitro upper gastrointestinal digestion [22]. The considerable changes in chemical composition caused by in vitro upper gastrointestinal tract digestion are likely attributed to the small intestine digestion rather than the gastric digestion because polyphenols are normally highly sensitive to mild-alkaline

Table 1 Chemical composition of in vitro upper gastrointestinal tract digested (DPME) and fermented (FPME) fractions of *Polygonum maritimum* aerial part ethanol extract determined by liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry (LC-DAD-ESI/MS^n)

No.	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg/g)	
						DPME	FPME
1	5.74	280	865	739(83),695(100), 577(74),575(47), 425(12),289(10),287(8)	β-type (epi)catechin trimer [47]	0.033 ± 0.001	ND
2	6.72	281/311	289	245(35),203(15), 137(29)	(+)-catechin [47]	0.038 ± 0.000	ND
3	7.77	280	865	739(74),695(100), 577(36),575(62), 425(15),289(12),287(14)	β-type (epi)catechin trimer [47]	0.044 ± 0.001	ND

Rt, retention time; λ_{max}, wavelengths of maximum absorption in the UV–Vis region; $[M-H]^-$, main ion; MS^2, fragment ions; ND, non-detected

conditions but mostly stable under acidic conditions. For instance, the composition of a raspberries (*Rubus idaeus* L.) leaf extract, which included phenolic compounds such as flavonol glycosides (e.g., quercetin and kaempferol derivatives), catechin, epicatechin, procyanidin B1, and phenolic acids, remained mostly stable after gastric digestion but was severely affected by small intestine digestion, where the total phenolics content decreased from 40.9 mg/g DW to 21.7 mg/g DW [48]. In light of the known stability of polyphenols in the stomach and potential partial degradation in the small intestine, myricetin and quercetin glycosides or their aglycones were expected to be detected in DPME. However, these flavonols were absent in DPME, possibly due to the limited solubility attributed to these compounds [49, 50]. In addition, analytical methodology and concentrations below the detection limits may contribute to their absence, including phenolic acid and oligomeric procyanidins.

Conventional polyphenol-rich foods (e.g., broccoli, apple pomace, pomegranate juice, onions) subjected to in vitro simulated upper gastrointestinal digestion have also demonstrated a trend of degradation of polyphenols mostly after small intestine conditions (e.g., epicatechin, procyanidin, quercetin-3-*O*-galactoside) [51]. Although catechin and (epi)catechin trimers were also susceptible to the conditions of the small intestine, as shown by the evident decrease in DPME (Table 1), a small number of compounds was still available to pass to the colon. The presence of flavan-3-ols in the colon may be related to the reported higher stability of these compounds, particularly their oligomers (procyanidins), under gastric and small intestine digestion conditions [52]. These compounds may be degraded to low-molecular-weight compounds by colonic microbiota [53]. Monomeric and oligomeric flavan-3-ols present in DPME were not detected in FPME. In line with this, the considerable degradation of compounds in a raspberries (*R. idaeus*) leaf extract after small intestine conditions increased notoriously after colonic fermentation [48]. Although the original compounds of the raspberries leaf extract were almost totally degraded (below the detection limit) after colonic fermentation, new compounds were detected and these probably resulted from the breakdown of compounds by microbiota [48]. Overall, these results show that the polyphenolic composition of PME was significantly altered by in vitro simulated upper gastrointestinal digestion and colonic fermentation. Consequently, the biological activities of PME may also be affected by digestion. Hence, the assessment of bioactivities of digested and fermented fractions is crucial to predict potential beneficial effects along the gastrointestinal tract. It should be noted that polyphenols that passed through the dialysis membrane represent compounds that would be absorbed by small intestinal cells in vivo and may partially justify the absence of compounds in DPME. These absorbed compounds were not addressed in the present study, with the focus being on the fraction of the extract that remains unabsorbed and becomes available for colonic fermentation. In addition, the in vitro digestion of PME was made in the absence of any food matrix. Therefore, these results do not account for potential interferences from concomitant food intake, and further studies should be done to assess this aspect. For comparison of biological activities between the ethanol extract and its digested and fermented fractions, concentrations expressed in µg GAE/mL instead of mass per volume allows more suitable comparisons because concentrations are estimated based on the reducing capacity of their phenolic compounds.

Antioxidant activity

Antioxidant properties provided by polyphenols could play a role in the prevention of oxidative stress-related diseases. In vitro simulated digestion significantly altered the phenolic composition of PME (Table 1), which may affect its antioxidant properties, as these are strongly dependent on chemical structure [54]. Therefore, the antioxidant activity of PME and its in vitro digested and fermented fractions

were investigated by measuring intracellular ROS levels under H_2O_2-induced oxidative stress using the redox-sensitive probe H_2DCFDA in HepG2 cell line. This cell line retains the activity of phase I and II xenobiotic-metabolizing enzymes, allowing oxidative stress–related metabolites (e.g., ROS) to be formed as a result of metabolic activation. Thus, HepG2 is a suitable model for measuring ROS production and antioxidant effects. The experimental conditions selected for this assay were previously tested in viability assays, confirming absence of toxicity (data not shown). Cells were pre-treated with five different concentrations of PME (0.313–5 µg GAE/mL), DPME (0.156–2.5 µg GAE/mL) or FPME (0.078–1.25 µg GAE/mL) for 20 h and exposed to 1 mM H_2O_2 for 20 min (Fig. 1). The selected concentrations of the samples were also tested in parallel (controls of samples; data not shown).

Cells only treated with the samples showed fluorescence similar to the negative controls, indicating that the selected concentrations of PME, DPME or FPME caused no effect on intracellular redox status (data not shown). Treatment with 1 mM H_2O_2 (positive control) significantly increased the fluorescence in comparison with the negative control ($p<0.05$), indicating higher intracellular ROS levels (Fig. 1). Pre-treatment with PME (Fig. 1A; $p<0.001$ and $p<0.0001$), DPME (Fig. 1B; $p<0.0001$) or FPME (Fig. 1C; $p<0.05$ and $p<0.0001$) significantly reduced intracellular ROS levels induced by H_2O_2 with a concentration dependency trend, revealing an antioxidant effect that persisted after simulated digestion. The antioxidant capacity of PME was noticeably strong, as the highest concentration tested (5 µg GAE/mL) caused a 64.1% reduction in ROS levels in comparison with the positive control. The antioxidant capacity decreased slightly after in vitro digestion of the upper gastrointestinal tract and considerably after colonic fermentation. For instance, 1.25 µg GAE/mL PME, DPME or FPME induced a reduction of intracellular ROS levels of 49.6%, 41.7% and 22.3%, respectively, when compared with the positive control.

The remarkable antioxidant properties of PME shown by its capacity to reduce intracellular ROS levels under oxidative stress in HepG2 cells (Fig. 1A) can result from the activation of antioxidant defense mechanisms by polyphenols (flavan-3-ols and flavonols; [1]), as a consequence of pre-treatment with the extract for 20 h before exposure to H_2O_2. In addition, ROS scavenging activity of polyphenols may contribute to the antioxidant effect during simultaneous exposure with PME and H_2O_2 (20 min). PME capacity to neutralize ROS has been previously suggested as a potential protection mechanism towards H_2O_2-induced death in *Saccharomyces cerevisiae* and likely results from its reported high content in phenolic compounds [7]. Moreover, PME displayed a high antiradical activity towards 2,2-diphenyl-1-picrylhydrazyl radical (DPPH; $EC_{50}=2.29\pm0.10$ µg/mL) [7]. Other authors have also documented the antioxidant properties of *P. maritimum* extracts towards H_2O_2 in cell-free [55] and cell-based assays [8]. Interestingly, a *P. maritimum* leaf methanol extract prevented H_2O_2-induced cytotoxicity in co-treatment but not in a 24 h pre-treatment in SH-SY5Y neuroblastoma cells [8]. The absence of protection towards H_2O_2 in the latter case, in opposition to our study, may be related with the use of different plant organs and/or cellular models. On the other hand, dichloromethane

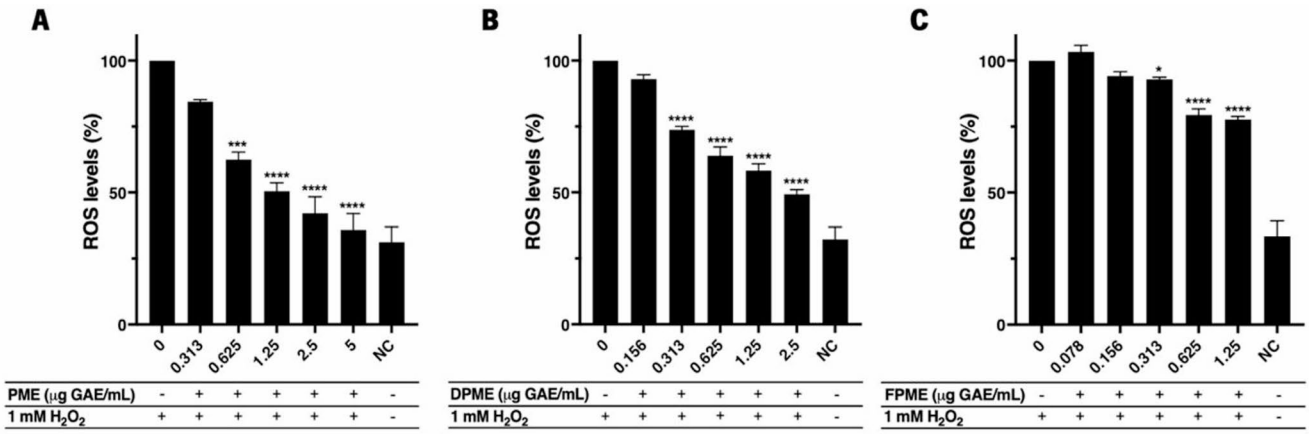

Fig. 1 Antioxidant activity of *Polygonum maritimum* ethanol extract (PME) persisted after in vitro digestion. HepG2 cells were pre-treated with PME (**A**), the in vitro upper gastrointestinal tract digested (DPME; **B**) or colonic fermented (FPME; **C**) fractions for 20 h and exposed to 1 mM H_2O_2 for 20 min. Cells treated with 1 mM H_2O_2 represented the positive control. The fluorescence emitted using 2',7'-dichlorodihydrofluorescein diacetate was measured to estimate intracellular ROS levels (%). Data are presented as the mean of three independent experiments normalized to the positive control [1 mM H_2O_2; respective non-normalized data were 138244.7±7983.1 (A), 152076.5±7709.8 (B), and 149136.5±12611.5 (C) arbitrary units] ± standard error of the mean. Statistically significant differences were investigated between the means of the positive control and the co-treatments using one-way ANOVA and Dunnett's multiple comparison test; (*) $p<0.05$, (***) $p<0.001$, (****) $p<0.0001$. NC, negative control (contained the solvents of the tested components); GAE, gallic acid equivalents; ROS, reactive oxygen species

leaf extract protected SH-SY5Y cells in both types of treatment, probably because this extract contains bioactive compounds with low polarity that cannot be extracted with methanol [8]. Overall, the antioxidant properties of *P. maritimum* extracts may arise from direct or indirect antioxidant mechanisms to neutralize ROS and other free radicals.

Degradation of phenolic compounds caused by digestion may affect the efficacy of antioxidant activity. Generally, there seems to be a predominant trend of loss of phenolic compounds after in vitro digestion that is frequently accompanied by a reduction of antioxidant activity, but the remaining activity may still be considered relevant and beneficial [20, 56, 57]. For instance, in vitro simulated upper gastrointestinal digestion of an ethanol: water (57:43, *v/v*) açaí (*Euterpe oleracea* Mart.) seed extract caused considerable changes in the amounts of catechin, epicatechin and procyanidins B1 and B2 and also in the antioxidant properties, decreasing the scavenging capacity of the ROS superoxide anion and hypochlorous radical and increasing the capacity to scavenge peroxyl radical [58]. In our study, the antioxidant activity of PME (Fig. 1A) was not considerably altered after in vitro upper gastrointestinal tract digestion (Fig. 1B) despite the noticeable degradation of compounds presented in DPME (Table 1). In contrast, the antioxidant activity of the extract decreased after colonic fermentation (Fig. 1C), in accordance with severe degradation of compounds observed in FPME (Table 1), with the effect being less pronounced and statistically significant only at the highest concentrations. This result suggests the presence of bioactive non-phenolic compounds undetected with the methodology used in the chemical analysis.

Studying the stability of phenolic compounds and the persistence of the antioxidant effect after digestion is of great importance because only those antioxidants that are not degraded in digestion become bioaccessible to be absorbed and induce the desired biological effect in the target tissue. Moreover, phenolic compounds that exhibit antioxidant activity along the gastrointestinal tract but are not locally absorbed can also contribute to health effects as they can protect the gastrointestinal tract against oxidative stress by acting as ROS scavengers [18].

Anti-inflammatory activity

Antioxidant and anti-inflammatory properties of polyphenols have been linked to their chemopreventive potential. Considering that in vitro digestion considerably changed the chemical composition of PME (Table 1) and affected its antioxidant capacity along the digestive tract (Fig. 1), the anti-inflammatory activity of PME and the impact of digestion on this bioactivity should also be assessed. The capacity to reduce the production of the pro-inflammatory mediator ·NO by LPS-stimulated macrophages is considered a measurement of anti-inflammatory potential. Thus, PME, DPME and FPME capacities to inhibit 50% of ·NO (EC_{50}) produced by LPS-stimulated RAW 264.7 macrophages were investigated and reported as >60 µg GAE/mL, 6.85 ± 0.29 µg GAE/mL and 8.37 ± 0.28 µg GAE/mL, respectively. The lower EC_{50} values of DPME and FPME in comparison with PME indicate that the digested and fermented fractions display higher anti-inflammatory activity. The anti-inflammatory potential of *P. maritimum* has been previously reported for dichloromethane [10] and acetone [9] extracts of leaves collected in Southern Portugal, showing EC_{50} values (48 µg/mL and 22.0 µg/mL, respectively) lower than that of PME (>167 µg/mL, corresponding to >60 µg GAE/mL), which indicates a higher anti-inflammatory activity for these extracts reported in the literature. These differences in activity are likely related to the use of different solvents that may have extracted compounds with higher anti-inflammatory potential than those found in PME, for instance, β-sitosterol, stigmasterol, 1-octacosanol, oleamide, linolenic and oleic acids [10].

Toxic chemicals consumed in the diet likely induce inflammation in the gastrointestinal tract, which can lead to chronic inflammation and, possibly, increasing the risk of cancer. The consumption of products that exhibit anti-inflammatory effects after digestion could result in a preventive role in the progression of inflammatory diseases. However, changes in the chemical composition of polyphenol-rich products induced by digestion also seem to decrease their anti-inflammatory activity. For instance, in vitro upper gastrointestinal digestion of *Vitis vinifera* L. leaf water extract, containing flavanols, anthocyanins and caffeic acid derivatives, considerably led to the degradation of compounds and drastically decreased its anti-inflammatory effects regarding the modulation of the expression of pro-inflammatory mediators in Caco-2 cells [59]. On the other hand, açaí (*E. oleracea*) seed extract subjected to simulated gastrointestinal digestion revealed decreased anti-inflammatory activity, but still remarkable, by reducing NF-kB activation and tumor necrosis factor α (TNF-α) levels in LPS-stimulated RAW 264.7 macrophages, which is in line with the previously mentioned changes in chemical composition (e.g., catechin, epicatechin and procyanidins B1 and B2) and antioxidant activity [58]. In our study, changes in the chemical composition of PME caused by simulated digestion led to a surprising increase of anti-inflammatory potential in DPME and FPME, suggesting beneficial effects towards inflammation in the colon. Undetected compounds activated by chemical changes during in vitro digestion might account for this anti-inflammatory effect.

Antimutagenicity

In addition to antioxidant and anti-inflammatory properties, antimutagenicity can have a pivotal role in the prevention of cancer initiation. Nowadays this bioactivity is particularly important because foods are commonly found contaminated with environmental or non-intended added substances, such as the mutagenic agent BaP, increasing cancer risk. Polyphenol-rich products with the capacity to inhibit mutagenicity could participate in the mitigation of the toxic effects of food mutagens. The antimutagenic effects of PME, DPME or FPME towards BaP, metabolically activated with S9 microsomal fraction of Sprague-Dawley rats, were assessed using the histidine-dependent *S. typhimurium* strains TA98 and TA100 (frameshift and base-pair substitution mutations, respectively) in the Ames test (traditional version for PME and miniaturized version for DPME and FPME). Bacteria were co-treated with three concentrations of PME (1.25, 2.5 or 5 µg GAE/plate), DPME (25, 50 or 100 ng GAE/well) or FPME (6.25, 12.5 or 25 ng GAE/well) and BaP (traditional: 3 µg/plate; miniaturized: 10 or 60 ng/well for TA98 and TA100 strains, respectively) and the number of colonies prototrophic for histidine were assessed (Table 2).

The number of revertant colonies exposed to different concentrations of the samples (PME, DPME or FPME) was comparable to those in the respective negative controls (data not shown), indicating absence of mutagenic effect. Treatment with BaP significantly increased the number of revertant colonies in comparison with the negative controls, confirming its recognized mutagenic effect (Table 2). PME significantly decreased the number of BaP-induced revertant colonies at 1.25, 2.5 and 5 µg GAE/plate in TA98 strain and at 2.5 and 5 µg GAE/plate in TA100 strain, with the inhibition of mutagenicity in the former and latter strains being up to 56% ($p<0.001$) and 74.6% ($p<0.0001$), respectively (Table 2). Therefore, PME seems to cause a concentration-dependent antimutagenic effect regarding frameshift and base-pair substitution mutations, and the protection against the latter type of mutation being stronger than the former type.

After in vitro digestion, DPME inhibited BaP-induced mutagenicity up to 55.2% ($p<0.001$) and 53.8% ($p<0.01$) in TA98 and TA100 strains, respectively, suggesting persistence of antimutagenic effect towards the two types of mutations until the extract reaches the colon. FPME did not significantly inhibit BaP-induced mutagenicity in the range of concentrations tested in TA98 strain and only caused a significant inhibitory effect of 48.8% at the highest concentration tested in TA100 strain. Hence, the metabolization of the extract by colonic microbiota affected its antimutagenic capacity, only protecting against base-pair substitution mutations. Interestingly, 25 µg GAE FPME/well induced a slightly higher antimutagenic effect towards base-pair substitution mutations (48.8%) in comparison with the same concentration of DPME (40.6%), suggesting that the activity towards this type of mutation was not negatively affected by colonic metabolization. These results suggest that PME can protect against frameshift and base-pair substitution

Table 2 Antimutagenicity of *Polygonum maritimum* ethanol extract (PME) and respective in vitro upper gastrointestinal tract digested (DPME) and colonic fermented (FPME) fractions towards benzo[a]pyrene (BaP) in *Salmonella typhimurium* TA98 and TA100 strains using the Ames test

	Number of revertants per plate/well (Mean ± SEM)		Inhibition of mutagenicity (%)	
	TA98	TA100	TA98	TA100
Treatment (µg/plate)				
Negative control	31.5 ± 4.0	143.0 ± 7.0	–	–
3 BaP	60.7 ± 1.7	499.7 ± 23.1	0	0
1.25 GAE PME + 3 BaP	46.0 ± 1.7*	470.7 ± 4.7	24.2	5.8
2.5 GAE PME + 3 BaP	34.7 ± 3.5**	244.0 ± 12.3****	42.9	51.2
5 GAE PME + 3 BaP	26.7 ± 3.5***	126.7 ± 5.4****	56.0	74.6
Treatment (ng/well)				
Negative control	1.73 ± 0.11	9.6 ± 0.99	–	–
10/60 BaP	14 ± 0.75	62.7 ± 5.27	0	0
25 GAE DPME + 10/60 BaP	8 ± 0.75**	37.3 ± 2.0**	42.9	40.6
50 GAE DPME + 10/60 BaP	7.2 ± 0.66**	33.0 ± 1.39**	48.6	47.4
100 GAE DPME + 10/60 BaP	6.27 ± 0.87***	29.0 ± 4.12**	55.2	53.8
6.25 GAE FPME + 10/60 BaP	15.6 ± 1.23	50.57 ± 4.57	-11.4[a]	19.4
12.5 GAE FPME + 10/60 BaP	12.0 ± 0.09	45.2 ± 6.50	14.3	28.0
25 GAE FPME + 10/60 BaP	9.08 ± 1.70	32.13 ± 2.27*	35.1	48.8

BaP used in this test was metabolically activated with +S9 fraction. The Ames test (traditional or miniaturized versions carried out in triplicate or in three independent experiments, respectively). The number of revertant colonies were counted in each treatment and the percentage of inhibition of mutagenicity was calculated. Statistical difference was assessed between positive controls and respective co-treatments using one-way ANOVA and Dunnett's multiple comparison test; $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****). [a]Negative value resulted from a higher number of revertant colonies in the co-treatment than in the positive control. GAE, gallic acid equivalents

mutations along the gastrointestinal tract, but after colonic fermentation the effect may be altered or inactivated. Although to the best of our knowledge no reports regarding the antimutagenicity of *P. maritimum* extracts were found in the literature, polyphenols related to those found in PME such as oligomeric flavan-3-ols (procyanidins isolated from grape seeds) were shown to decrease the number of revertants caused by BaP in TA100 strain in the Ames test [60]. Since monomeric and oligomeric flavan-3-ols were detected after in vitro upper gastrointestinal tract digestion, the antimutagenic effect of DPME may result from the activity of these compounds that remained in the extract.

Antigenotoxicity

Although the liver is the primary organ for metabolization and detoxification of food contaminants like BaP, epithelial cells of the gastrointestinal tract are firstly exposed to these toxic chemicals [61] and can metabolize them to some extent, potentially activating their toxicity and leading to DNA damage that increase cancer risk. Accordingly, ingestion of BaP-contaminated foods has been associated with increased colon cancer risk [62]. Cellular models of colon and liver are suitable for studying DNA protective effects of polyphenol-rich plant extracts against BaP because they can activate the genotoxicity of procarcinogens due to the presence of xenobiotic-metabolizing enzymes [63] and represent potential sites of exposure during digestion. HepG2 cell line has been used as a cellular model to study the toxicity of genotoxic procarcinogens as these liver cells retain the activity of phase I and II xenobiotic-metabolizing enzymes. Caco-2 cell line also retains phase I enzyme CYP1A1 and phase II enzymes GSTs, UDP-glucuronosyltransferases (UGTs) and sulfotransferases [63]. Considering that reactive metabolites and ROS are generated from BaP metabolization and both can induce DNA damage, the capacity of PME to protect from the genotoxicity of BaP and an oxidative stress-inducing agent can highlight its potential role as cancer preventive agent. Thus, the antigenotoxic effects of PME and its digested and fermented fractions were evaluated against BaP- and H_2O_2-induced DNA damage in HepG2 and Caco-2 cells using the comet assay. The conditions tested in these experiments were selected considering results of viability assays (PrestoBlue high sensitivity cell viability reagent) in HepG2 and Caco-2 cells that showed absence of toxicity (data not shown) and non-genotoxic concentrations of PME, DPME and FPME (data not shown). Cells were pre-treated with PME (1.25, 2.5 or 5 µg GAE/mL), DPME (0.625, 1.25 or 2.5 µg GAE/mL) or FPME (0.313, 0.625 or 1.25 µg GAE/mL) for 20 h and subsequently exposed to 200 µM or 100 µM H_2O_2 for 30 min in HepG2 (Fig. 2A-C) and Caco-2 (Fig. 2D-F) cell lines, respectively. The results showed that PME attenuated H_2O_2-induced DNA damage (comet score) at all tested concentrations (Fig. 2A; $p<0.05$ and $p<0.01$), whereas DPME caused no significant effect (Fig. 2B) and FPME only protected DNA at the highest concentration tested (Fig. 2C; $p<0.05$) in HepG2 cells. In addition, PME (Fig. 2D; $p<0.05$, $p<0.01$, and $p<0.001$) and DPME (Fig. 2E; $p<0.01$) significantly reduced oxidative DNA damage in Caco-2 cells, but FPME (Fig. 2F) caused no effect.

Similar experiments were performed with BaP to investigate the protective effect of the extract and its digested and fermented fractions against a genotoxic food contaminant. HepG2 (Fig. 3A–C) and Caco-2 (Fig. 3D–F) cells were co-treated with PME, DPME or FPME and 50 µM BaP for 20 h. The results show that PME (Fig. 3A, $p<0.05$ and $p<0.0001$; Fig. 3D, $p<0.05$ and $p<0.01$) and DPME (Fig. 3B and E; $p<0.01$) protected DNA from damage induced by BaP, but no significant effect was observed with FPME (Fig. 3C, F) in HepG2 and Caco-2 cells. Notably, DPME at 2.5 µg GAE/mL decreased the comet score from 100% (positive control, 50 µM BaP) to 82% and 74% in HepG2 and Caco-2 cells, respectively, suggesting that the extract provided higher DNA protection in Caco-2 cells. Altogether, these results show that PME exhibited antigenotoxicity towards oxidative stress- and BaP-induced DNA damage. These effects persisted in the colon-available fraction (DPME) in colon cells and thus suggesting that the ingested extract could provide DNA protection along the gastrointestinal tract until it reaches the colon. In the colon, the antigenotoxic effect seems to be inactivated after metabolization by microbiota, which correlates with the severe degradation of compounds observed in FPME profile by LC-DAD-ESI/MS^n (Table 1).

PME antigenotoxicity towards H_2O_2 in HepG2 and Caco-2 cells is in line with a previous report on DNA protective effect against H_2O_2-induced oxidative damage in the dominant deletion (dDEL) assay in *S. cerevisiae*, in which the protective mechanism was suggested to result from ROS scavenging capacity [7]. Nevertheless, the activation of antioxidant defense mechanisms is expected to play the major role in the antigenotoxic effect towards H_2O_2-induced damage observed in our study, as cells were pre-treated with the extract for 20 h prior to exposure to H_2O_2. This activation can refer to the modulation of the expression of antioxidant enzymes by the central protein nuclear factor-erythroid-2-related factor-2 (NRF2) that translocate to the nucleus and regulates the antioxidant-responsive elements (ARE) responsible for the expression of these enzymes [1, 64]. PME capacity to decrease intracellular oxidation of HepG2 cells challenged with H_2O_2 (Fig. 1A) seems to corroborate the proposed potential contribution of its strong antioxidant properties, at least partially, to its antigenotoxicity towards H_2O_2.

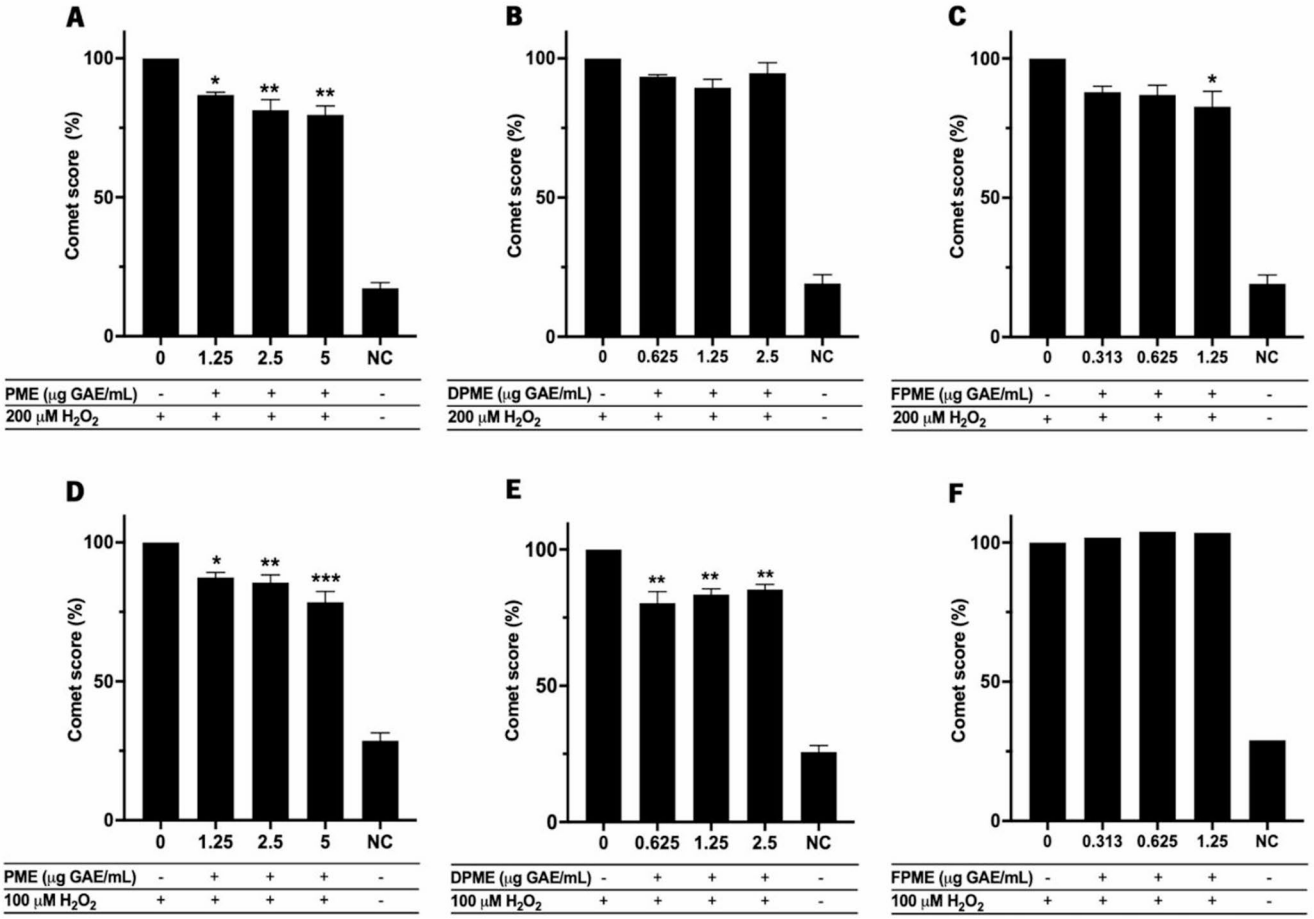

Fig. 2 Effect of *Polygonum maritimum* ethanol extract (PME) upon in vitro digestion on the protection towards oxidative stress-induced DNA damage. HepG2 (**A**–**C**) and Caco-2 (**D**–**F**) cells were pre-treated with PME (**A**, **D**), the in vitro upper gastrointestinal tract digested (DPME; **B**, **E**) or colonic fermented (FPME; **C**, **F**) fractions for 20 h and subsequently exposed to 200 µM or 100 µM H_2O_2, respectively, for 30 min. DNA damage was assessed using the comet assay and the data were expressed as comet score (%) normalized to positive control [non-normalized data for 200 µM H_2O_2 were 269.3 ± 4.4 (**A**) and 232 ± 7.4 (**B**, **C**); and for 100 µM H_2O_2 were 267.7 ± 10.1 (**D**), 289 ± 3.7 (**E**) and 203 (**F**)]. Data are presented as the mean of three (**A**–**E**) or one (**F**) independent experiments ± standard error of the mean. Statistical significance was assessed between the positive control and the treatments with one-way ANOVA and Dunnett's multiple comparison test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$(***). NC, negative control (contained the solvents of the tested components); GAE, gallic acid equivalents

Polyphenols play a role in the bioactivities of PME and, consequently, alterations in the phenolic composition such as those caused by digestion will possibly alter the beneficial effects attributed to the extract. Indeed, the chemical composition of PME was severely affected after simulation of digestion, which caused loss of effect in most bioactivities, except for the anti-inflammatory activity. The antioxidant effect of PME under oxidative stress was not severely affected after in vitro upper gastrointestinal tract digestion (Fig. 1B), but its antigenotoxic effect towards H_2O_2 was absent in DPME in HepG2 cells (Fig. 2B), suggesting that the persistence of antioxidant properties was not enough to provide an antigenotoxic effect. Surprisingly, FPME revealed antigenotoxic effects at the highest concentration tested against H_2O_2 in HepG2 cells (Fig. 2C), indicating protection towards oxidative DNA damage. Conversely, the antigenotoxic effect of PME towards H_2O_2 persisted after in vitro simulated upper gastrointestinal tract digestion in Caco-2 cells, which can potentially involve an antioxidant effect as suggested for the in vitro digested polyphenol-rich extract of *Ginkgo biloba* L. in the human colonic adenocarcinoma cell line HT29 [19]. The differences in the antigenotoxicity against H_2O_2 in HepG2 and Caco-2 cells could be the consequence of the different antioxidant/detoxifying systems and metabolic enzyme activities between these two cell lines. Other polyphenol-rich products also revealed changes in the chemical composition after in vitro digestion but still presented persistent antigenotoxicity, such as the case of açaí (*E. oleracea*) pulp in which only 49.8% of the total initial polyphenols were available for colonic fermentation; however, despite of these changes, the antigenotoxic properties remained relevant throughout the entire digestive

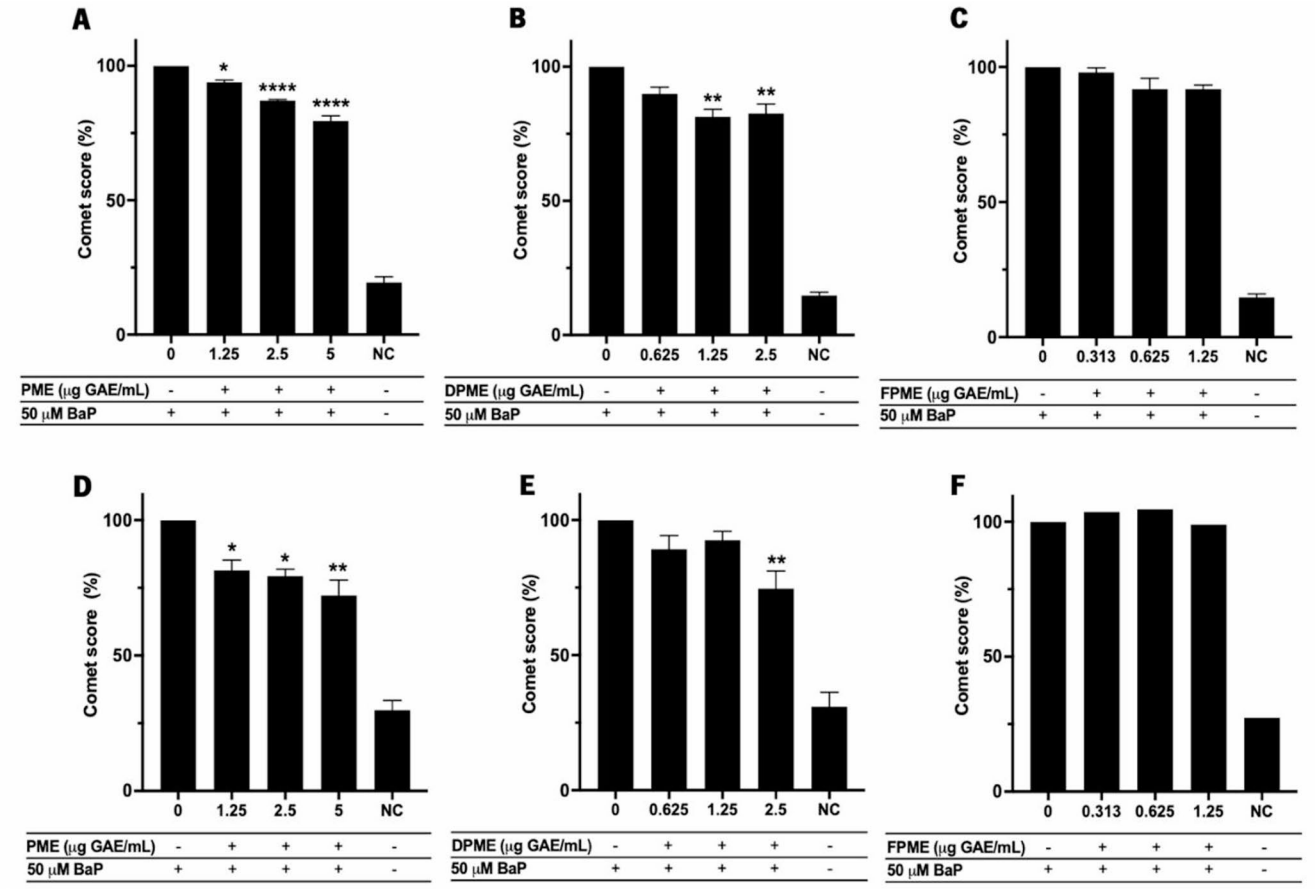

Fig. 3 Persistence of antigenotoxicity of *Polygonum maritimum* ethanol extract (PME) against benzo[a]pyrene (BaP) after simulated digestion of the upper gastrointestinal tract. HepG2 (**A**–**C**) and Caco-2 (**D**–**F**) cells were co-treated with PME (**A**, **D**), the in vitro upper gastrointestinal tract digested (DPME; **B**, **E**) or colonic fermented (FPME; **C**, **F**) fractions and 50 µM BaP for 20 h, and DNA damage was investigated by comet assay. Data were expressed as comet score (%) normalized to the positive control [50 µM BaP; respective non-normalized data were 279.7 ± 1.4 (**A**), 261.7 ± 3.1 (**B**, **C**), 205 ± 11.4 (**D**), 180.7 ± 5.9 (**E**), 194 (**F**)] and represents the mean value of three (**A**–**E**) or one (**F**) independent experiments ± standard error of the mean. Statistical significance was determined between the positive control and the treatments with one-way ANOVA and Dunnett's multiple comparison test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.0001$ (****). NC, negative control (contained the solvents of the tested components); GAE, gallic acid equivalents

process in HT29 cells [65]. Therefore, PME is suggested to display antigenotoxic properties under oxidative stress generated along the gastrointestinal tract, at least until it reaches the colon. Potential DNA protection in the colon cannot be entirely excluded because phenolic compounds may cause a local effect before metabolization by colonic microbiota. An antigenotoxic effect under oxidative stress in colon cells can be of extreme relevance, as it could pose a line of defense towards ROS-induced DNA damage caused by exogenous sources such as toxic contaminants frequently ingested in the diet.

The human mutagen and carcinogen BaP caused genotoxicity in HepG2 (Fig. 3A–C) and Caco-2 (Fig. 3D–E) cells and mutagenicity in *S. typhimurium* (Table 2). In both cases, PME was able to attenuate the effects of BaP on DNA. These results clearly show that polyphenol-rich extracts like PME are promising candidates to include in the human diet to attenuate genotoxicity and mutagenicity, and potential carcinogenicity, caused by foods contaminated with BaP. Notably, the antigenotoxic properties of PME against BaP in the liver (Fig. 3A) and colon (Fig. 3D) cells persisted after in vitro simulation of upper gastrointestinal tract digestion (Fig. 3B and E) but were inactivated by colonic fermentation (Fig. 3C, F) in similarity to the antimutagenicity results in *S. typhimurium* (Table 2). Thus, the antigenotoxic effects of PME may be active along the digestive tract but probably will be limited in the colon. Antigenotoxic and antimutagenic mechanisms of PME against BaP may include direct interaction of polyphenols with BaP, interfering with its bioactivation and avoiding it; inhibition of phase I xenobiotic-metabolizing enzymes responsible for BaP bioactivation (e.g., CYP1A1) that generate genotoxic metabolites; induction of phase II xenobiotic-metabolizing enzymes and antioxidant effects that can participate in the inactivation

of genotoxic metabolites generated from BaP bioactivation (reactive metabolites and ROS, respectively [66]). Particularly, inhibition of BaP bioactivation due to the downregulation of *CYP1A1* expression is often suggested as one of the main causes of the antigenotoxic effects of polyphenol-rich products [67–69], and it could be related to the inhibition of AHR activation and translocation to the nucleus, where it can regulate the expression of cytochrome P450 genes [69]. The capacity of polyphenols to interfere in signaling pathways involved in the detoxification of BaP can also be considered, such as the case of quercetin which induced the translocation of AHR and NRF2 to regulate the expression of enzymes that can participate in the detoxification of BaP [70]. Hence, the role of AHR seems to be fundamental in the metabolization of BaP and its activation can have distinct roles, either mediating bioactivation of BaP and increasing genotoxicity or by participating in the induction of detoxifying enzymes, along with NRF2, to inactivate BaP genotoxic metabolites.

Relative expression of *CYP1A1*

Considering that downregulation of *CYP1A1* expression could be involved in the protection against BaP-induced DNA damage, the effect of co-treatment of PME (1.25, 2.5 or 5 µg GAE/mL), DPME (0.625, 1.25 or 2.5 µg GAE/mL) or FPME (0.313, 0.625 or 1.25 µg GAE/mL) and 1 µM BaP for 20 h on the relative expression of *CYP1A1* was assessed using RT-qPCR in HepG2 and Caco-2 cells. The results showed that there was no statistical difference between the relative expression of *CYP1A1* observed in the positive control and in the co-treatments with PME, DPME or FPME and BaP in HepG2 (Fig. 4) and in Caco-2 (data not shown) cells. Still, the lowest concentration tested of PME (1.25 µg GAE/mL; Fig. 4A), DPME (0.625 µg GAE/mL; Fig. 4B) and FPME (0.313 µg GAE/mL; Fig. 4C) showed *CYP1A1* expression noticeably lower than that of the positive control in HepG2 cells in a consistent manner, although without significance, suggesting a potential trend of inhibition of *CYP1A1* expression for concentrations of sample lower than those tested in this experiment. In addition, these results suggest that only low concentrations of sample may be required for induction of a potential protective mechanism towards BaP.

Higher concentrations of the samples, which induced an antigenotoxic effect in the comet assay (2.5 and 5 µg GAE/mL for PME, Fig. 3A; and 1.25 and 2.5 µg GAE/mL for DPME, Fig. 3B), were not capable of inducing a significant effect on BaP-induced *CYP1A1* expression, implying that other mechanisms may be involved in the antigenotoxic effect. In opposition to our study, extracts rich in phenolic compounds have been indicated to significantly downregulate the expression of *CYP1A1*, such as the case of an extract of black soybean seed coat [*Glycine max* (L.) Merr] in which this effect was suggested to result from the inhibition of the transformation of AHR and to be one of the main responsible mechanisms for the antigenotoxic effect against BaP in HepG2 cells [69]. Considering the results obtained in our study, inhibition of *CYP1A1* expression may not be the mechanism responsible for the antigenotoxicity provided by PME (Fig. 3A, D) and DPME (Fig. 3B, E) in HepG2 and Caco-2 cells. Possibly, this antigenotoxic effect

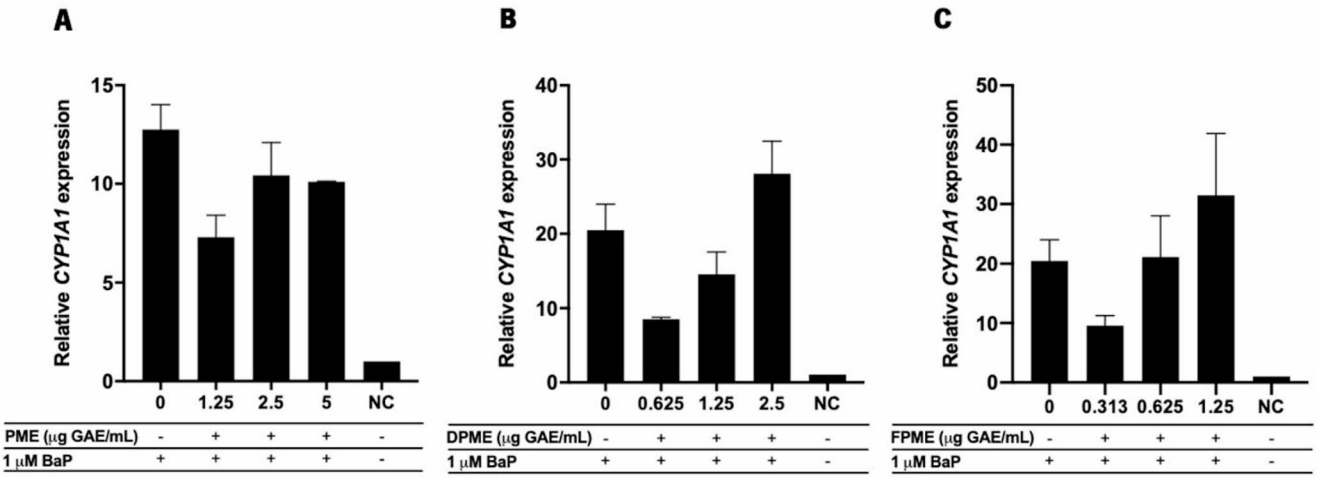

Fig. 4 Benzo[a]pyrene (BaP)-induced *CYP1A1* expression was not significantly affected by treatment with *Polygonum maritimum* ethanol extract (PME) or the in vitro digested (DPME) and fermented (FPME) fractions. HepG2 cells were co-treated with PME (**A**), DPME (**B**) or FPME (**C**) and 1 µM BaP for 20 h. Relative expression of *CYP1A1* was determined by normalizing the data to the expression of the reference gene *GAPDH* and to results of untreated cells (respective relative expression considered 1). Data are presented as the mean of two independent RNA extractions and two reverse transcriptions from one extraction and one from another extraction ± standard error of the mean. Statistical difference was investigated between the positive control (1 µM BaP) and the co-treatment with one-way ANOVA. NC, negative control (corresponds to untreated cells); GAE, gallic acid equivalents

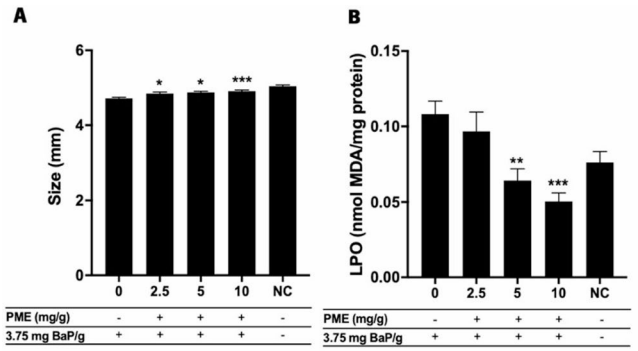

Fig. 5 Protective effects of *Polygonum maritimum* ethanol extract towards benzo[a]pyrene (BaP) in zebrafish (*Danio rerio*). Larvae were fed with only 3.75 mg BaP/g diet (positive control) or in co-treatment with 2.5, 5 or 10 mg PME/g diet and 3.75 mg BaP/g diet from 6–16 days-post fertilization. Diet containing only the solvents of the tested components represented the negative control (NC). Size (**A**) was measured and lipid peroxidation (LPO; **B**) was determined by spectrophotometry. Data are expressed as the mean ± standard error of the mean ($n = 8$). Statistical difference was assessed between the positive control and the co-treatments with Kruskal Wallis test (**A**) or one-way ANOVA and Dunnett's multiple comparison test (**B**); $p < 0.05$ (*), $p < 0.01$(**), $p < 0.001$(***). MDA, malondialdehyde

could be related with the modulation of NRF2/ARE signaling pathway, inducing detoxifying enzymes capable of inactivating the genotoxic metabolites. Moreover, the possibility that polyphenols may have interacted with the genotoxic metabolites, avoiding their direct contact with DNA and subsequent DNA adduct formation, should also not be discarded as a potential protective mechanism.

In vivo protective effects

The protective effects of PME towards BaP and their persistence after in vitro simulated digestion were shown in cellular models. Considering the digestion, absorption and metabolization that occur in the human body, a multicellular model organism was selected to assess the beneficial effects of ingesting PME in the presence of BaP. Zebrafish (*Danio rerio*) is considered an alternative to mammalian models for toxicological studies, sharing over 70% homology with the human genome. Therefore, an experiment was performed with zebrafish larvae by feeding a diet containing PME (2.5, 5 or 10 mg/g diet) and 3.75 mg BaP/g diet or a diet containing only BaP (positive control) from 6 to 16 dpf. Diets containing only the extract or the solvents of the tested components were also included in the experiment for control purposes (controls of sample and negative controls, respectively). At 16 dpf, protective effects of PME towards BaP regarding size of larvae and levels of MDA were determined (Fig. 5).

Larval survival rates were within normal values for the developmental stage used [71] (data not shown), with no significant differences between the negative control and the treatments. Feeding zebrafish with only 2.5, 5 or 10 mg PME/g diet caused no significant effect on size and MDA levels of larvae in comparison with the negative control (data not shown), indicating absence of toxicity. Diet containing BaP caused a 6% reduction in larvae size in comparison with the negative control, from 5.0 to 4.7 mm. The co-treatment of BaP with PME at 2.5, 5 or 10 mg PME/g diet attenuated this effect (Fig. 5A), suggesting that PME may overall be counteracting BaP-induced toxicity. One of the potential causes of this toxicity that led to reduced larvae size could be the induction of oxidative stress and related damage generated during BaP metabolization. Thus, the oxidative state of larvae was measured by determining the levels of MDA, a product from lipid peroxidation and a well-known marker of oxidative stress. The results show that BaP increased MDA levels by approximately 35% in comparison with the negative control (Fig. 5B). Co-treatment of BaP with the extract seems to have a dose-dependent effect, causing significant protection at 5 ($p < 0.01$) and 10 ($p < 0.001$) mg PME/g diet, which is possibly related to the antioxidant properties of PME that prevented LPO and may partially explain the protective effect regarding larvae size. These results are in line with the capacity of polyphenols, such as catechins, quercetin, and myricetin, to reduce BaP-induced oxidative stress [72]. Polyphenol-rich products (e.g., green or white tea, ethanol extract of *Cissampelos pareira* (L.) Hirsuta roots) fed to mice have also been shown to mitigate BaP toxicity related to oxidative stress, including LPO and other oxidative stress markers (e.g., levels of glutathione and activities of antioxidant enzymes [73, 74]). Protective effects of PME towards BaP-induced toxicity in a feeding experiment in the model organism zebrafish corroborates the beneficial effects observed in cellular models after in vitro simulated digestion.

Conclusion

The present study showed that the polyphenol-rich extract of the halophyte *P. maritimum* displays significant in vitro antioxidant, antigenotoxic, and antimutagenic properties and suggests potential protective effects in vivo. Therefore, the inclusion of this extract in the human diet could benefit human health. The digestion of polyphenol-rich products can alter the fate and efficacy of the proposed beneficial effects attributed to these products. Our results suggest that the chemical composition of *P. maritimum* extract is significantly altered by digestion, but the above-mentioned protective properties against contaminants-related toxicity are expected to persist along the digestive tract or, at least, until the colon is reached. To our knowledge, this is the first

in vitro prediction of the stability and bioaccessibility of a *P. maritimum* extract and respective assessment of biological effects after simulated upper gastrointestinal tract digestion and colonic fermentation. In addition, we presented evidence that ingested *P. maritimum* extract can provide in vivo protection against BaP-induced toxicity in zebrafish larvae co-fed with a diet containing the extract and the contaminant. Overall, our study suggests that the ingestion of *P. maritimum* may provide a potential chemopreventive effect in the gastrointestinal tract, emerging as a novel functional food ingredient for industrial application. This study contributes to the recognition of halophytes as a highly promising source of biofunctional compounds and products for human health.

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Author contributions Daniela Oliveira: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing; Ruzanna Hayrapetyan: Investigation, Methodology, Writing - review & editing; Maria Inês Dias: Formal analysis, Investigation, Methodology, Writing - review & editing; Maria João Rodrigues: Investigation, Methodology; Vanesa Gesser Correa: Investigation, Methodology; António Paulo Carvalho: Investigation, Methodology, Resources, Supervision; Ludovic Le Corre: Conceptualization, Funding acquisition, Methodology, Project management, Resources, Supervision, Writing - review & editing; Isabelle Séverin: Conceptualization, Funding acquisition, Methodology, Project management, Resources, Supervision, Writing - review & editing; Rosane Marina Peralta: Methodology, Resources, Supervision; Miguel Machado Santos: Conceptualization, Funding acquisition, Methodology, Project management, Resources, Supervision, Writing - review & editing; Luísa Custódio: Conceptualization, Funding acquisition, Methodology, Project management, Resources, Supervision, Writing - review & editing; Marie-Christine Chagnon: Conceptualization, Funding acquisition, Methodology, Project management, Resources, Supervision, Writing - review & editing; Rui Oliveira: Conceptualization, Funding acquisition, Methodology, Project management, Resources, Supervision, Writing - review & editing.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare no competing interests.

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