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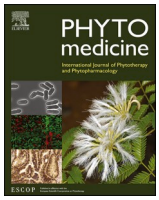

Original Article

Polyphenolic extracts from *Diospyros kaki* and *Vitis vinifera* by-products stimulate cytoprotective effects in bacteria-cell host interactions by mediation of transcription factor Nrf2

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ABSTRACT

Background: The intestinal and skin epithelium play a strong role against bacterial stimuli which leads to inflammation and oxidative stress when overwhelmed. Polyphenols from fruit-rich diets and by-products show promise against bacterial deleterious effects; however, their antibacterial and health-promoting effects remain understudied.

Purpose: This study aimed to assess the impact of polyphenolic extracts of grape (GrPE), persimmon (PePE) and pomegranate (PoPE) by-products on bacterial pathogen-host interactions, focusing beyond growth inhibition to explore their effects on bacterial adhesion, invasion, and modulation of host responses.

Methods: The microdilution method, as well as the tetrazolium based MTT cell proliferation and cytotoxicity assay with crystal violet staining were used to identify extracts sub-inhibitory concentrations that interfere with bacterial adhesion, invasion or lipopolysaccharides (LPS) effect on cell hosts without compromising host viability. The cytoprotective effects of extracts were assessed in a knock-down model of nuclear factor erythroid 2-related factor 2 (Nrf2).

Results: All extracts demonstrated significant reductions in pathogen adhesion to Caco-2 and HaCaT cells while preserving cellular integrity. Notably, PePE exhibited specific efficacy against *Salmonella enterica* adhesion, attributed mostly to its gallic acid content, whereas PoPE reduced *S. enterica* invasion in Caco-2 cells. The extracts supported the prevalence of non-pathogenic and commensal strains of intestinal and skin surfaces, selectively reducing pathogenic adhesion. The extracts mitigated the oxidative stress, enhanced the barrier function, and modulated the pro-inflammatory cytokines in LPS-challenged cells. GrPE, rich in anthocyanins, and PePE were found to mediate their protective effects through Nrf2 activation, while PoPE exerted multifaceted actions independent of Nrf2.

Abbreviations: ABTS$^{\bullet+}$, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ANOVA, analysis of variance; ATCC, American Type Culture Collection; Caco-2, human colorectal adenocarcinoma cells; CECT, Spanish Type Culture Collection; CLS, Cell Lines Services; CRISPR, clustered regularly interspaced short palindromic repeats; CV, crystal violet; DMEM, Dulbecco's modified eagle medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ELISA, enzyme linked immunosorbent assay; FESEM, field emission scanning electron microscopy; GrPE, polyphenolic extract of grape; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HaCaT, human keratinocytes; HHDP, galloyl-bis-hexahydroxydiphenoyl; IL-6, interleukin 6; IL-8, interleukin 8; KD, knockdown; KO, knockout; LB, Luria-Bertani medium; LC-MS/MS, liquid chromatograph mass spectrometer; LPS, lipopolysaccharides; MIC, minimum inhibitory concentration; MRM, multiple reaction monitoring; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear factor erythroid 2-related factor 2; OD_{600}, optical density measured at 600 nm; p/s, penicillin/streptomycin; PBS, phosphate buffered saline; PCA, principal component analysis; PePE, polyphenolic extract of persimmon; PoPE, polyphenolic extract of pomegranate; ROS, reactive oxygen species; SIC, sub-inhibitory concentration; TEER, transepithelial electrical resistance; TFC, total flavonoid content; TNF-α, tumour necrosis factor alpha; TPC, total phenolic content; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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Conclusion: Our results highlight the therapeutic potential of GrPE, PePE, and PoPE in shaping bacterial-host interactions, endorsing their utility as novel nutraceuticals for both oral and topical applications to prevent potential bacterial infections through innovative mechanisms.

Introduction

The intestinal and skin epithelium play an important role in protecting the human system against pathogenic bacteria as well as exogenous molecules stimuli (Ferrari et al., 2016; Gea-Botella et al., 2021). During the interaction of mammalian cells with pathogens, inflammation serves as the temporary and protective response of the immune system (Esteban-Fernández et al., 2018; Tanaka et al., 2018). When the interplay between mammalian cells and pathogens overwhelms the innate immune response, an excessive amount of reactive oxygen species (ROS) is accumulated in the mitochondria while uncontrolled release of proinflammatory mediators may initiate a chain of events. Thus, the disruption of cell functionality may lead to oxidative stress increase, barrier function loss, tissue damages and chronic inflammatory diseases (Kim et al., 2023; Tanaka et al., 2018; Yan et al., 2016).

Studies have underscored a strong correlation between a reduced incidence of pathologies and consumption of a diet rich in fruit and vegetable, which serve as bioactive compounds reservoirs (De Paula Menezes Barbosa et al., 2020; Parkar et al., 2014; Peres et al., 2015; Silva et al., 2016). Among them, polyphenolic compounds have undergone comprehensive research for their health-promoting effects encompassing antioxidant and anti-inflammatory activities (Kim et al., 2023; Nallathambi et al., 2020; Silva et al., 2016; Tanaka et al., 2018). Polyphenolics also exert antimicrobial properties; yet, most studies have predominantly emphasized their disruption capacity of cytoplasmic membranes as well as inhibition of enzyme activity (Alvarado-Martinez et al., 2020; Friedman et al., 2013; Manso et al., 2021; Moreno-Chamba et al., 2023; Silva et al., 2016), thereby leaving underexplored antimicrobial properties such as attenuating pathogenicity.

Polyphenolic compounds may exhibit a diverse array of mechanisms that interfere with microbial pathogenesis, potentially obstructing other phases of bacterial infections beyond their inhibitory properties (Esteban-Fernández et al., 2018; Gato et al., 2020; Kim et al., 2023; Li et al., 2014; Moreno-Chamba et al., 2023; Silva et al., 2016). Additionally, these compounds may boost the growth of health-promoting indigenous bacteria residing in the gastrointestinal and skin microbiome, enhancing their competitive advantage for binding sites (De Paula Menezes Barbosa et al., 2020; Peres et al., 2015). Furthermore, polyphenolic molecules can shield human cells from the deleterious effects of bacterial toxins such lipopolysaccharides (LPS), known to induce severe low-grade metabolic endotoxemia (Nallathambi et al., 2020). The effect of polyphenolics in LPS-stressed cells can down-regulate upstream signaling pathways associated with inflammation, reducing pro-inflammatory cytokine levels, and decreasing oxidative stress.

Studies have mentioned that polyphenolic molecules are able to modulate specific signaling proteins involved in the antioxidant adaptive response, rendering cells more resilient to the external stimuli like pathogens (Ferrari et al., 2016; Gasparrini et al., 2018; Kim et al., 2023; Nallathambi et al., 2020). In particular, the expression of genes regulated by nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that modulates cellular stress, plays a pivotal role in cellular adaptive processes. This has prompted increasing attention on the potential of polyphenolic extracts to induce adaptive cellular responses, activating genes encoding antioxidant proteins and detoxifying enzymes (Gasparrini et al., 2018; Ordonez et al., 2022; Reddy et al., 2009), shielding cell host from pathogenic stimuli.

Since industrial by-products of grape (*Vitis vinifera* L.), pomegranate (*Punica granatum* L.) and persimmon (*Diospyros kaki* Thunb.) serve as valuable sources of polyphenolic compounds with well-documented antibacterial activities (Esteban-Fernández et al., 2018; Luís et al., 2014; Moreno-Chamba et al., 2022; Romier-Crouzet et al., 2009; Silva et al., 2023; Xu et al., 2015), the mode of action of polyphenolic-rich extracts prepared from these by-products could hold potential promise for enhancing cellular defence mechanism against bacterial infections. By this way, the revalorization of these by-products could boost their economic and industrial relevance in Spain, increasing the utility of by-products form winemaking and juice-extraction sectors. The aim of the present research was to assess the cytoprotective potential of polyphenolic-rich extracts obtained from several fruit by-products such as persimmon, grape and pomegranate in mammalian cells exposed to pathogenic bacteria, at sub-inhibitory concentrations (SIC).

Material and methods

Microbiological, cell culture media and chemical reagents

Microbiological culture media were acquired from Scharlab (Barcelona, Spain), and human cell culture media and reagents were sourced from Gibco (Madrid, Spain). Antibiotics, chemicals, pure polyphenolic compounds, and reagent solutions were purchased from Merck (Madrid, Spain) and Panreac (Barcelona, Spain). Transfection reagents were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Plant material and polyphenolic extraction

Grape, persimmon and pomegranate by-products such as peels, skins, pulp and seeds were processed at pilot scale by Mitra Sol Technologies S.L., using vacuum-expansion technologies (Moreno-Chamba et al., 2023) (Supplementary plant material and phenolic extracts). Grape by-product was obtained from winemaking industry, while pomegranate and persimmon by-products were obtained from juice industry. After processing, three extracts were obtained: available polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE). Stock solutions of extracts were prepared at 60 mg/ml (based on dried weight of extract).

Analysis of polyphenolic composition of extracts; determination of total polyphenolic and flavonoid content and assessing of the antioxidant activity

A Liquid Chromatograph Mass Spectrometer system (LCMS-8050^{TM}, Shimadzu, Kyoto, Japan) was used for polyphenolic profile determination (Garrido et al., 2011; Jiménez-Sánchez et al., 2015; Mena et al., 2013, 2012; Salazar-Bermeo et al., 2021). The mobile phases, conditions and gradient used for LC-MS/MS analysis are shown in Supplementary material Table S1, while Supplementary material Table S2 summarizes the MS parameter settings. Polyphenolic identifications were performed by multiple reaction monitoring (MRM) using pure polyphenolic compounds as marker compounds. The limit of quantification (LOQ) for each compound was determined based on the lowest consistent and measurable area values and converted to μg/ml for clarity. The marker compounds and more details for quantification are described in Supplementary LC-MS/MS method details. The MS parameters (precursors and product ions for MRM transitions, corresponding to optimized collision energy and retention time for each compound) are listed in Supplementary material Table S3.

Total phenolic content (TPC) of the samples was determined using Folin-Ciocalteau assay and total flavonoid content (TFC) was determined using aluminium-chloride method. The extracts antioxidant

activity was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical assays, according to (Salazar-Bermeo et al., 2023). Absorbance of samples of each reaction were recorded by a microplate reader (Cytation™ 3 Cell Imaging Multi-Mode, BioTek, Winnoski, VT, USA).

Mammalian and bacterial cells

Human colorectal adenocarcinoma cells (Caco-2) and human keratinocytes (HaCaT) cells were procured from ATCC (Manassas, VA, USA) and CLS (Eppelheim, Germany), respectively. Both cell lines were incubated (37 °C under 5 % CO_2) and maintained in their respective culture media (Supplementary material Table S4). Cells between 5–40 passages were used for assays. The selection of both cell lines was based on their ability to mimic intestinal and skin epithelia, respectively, making them ideal for studying interactions between bacterial strains and the intestinal or skin epithelium, simulating the barrier properties during interactions with bacterial strains (Birhanu et al., 2021; Gea-Botella et al., 2021; Jeng and Yan, 2022; Kim et al., 2023; Romier-Crouzet et al., 2009).

Bacterial strains were purchased from the CECT (Valencia, Spain). For the interaction of bacteria with Caco-2 cells, *Klebsiella pneumoniae* subsp. *pneumoniae* CECT 143 and *Salmonella enterica* serovar Typhimurium CECT 443 were used, due to their association with intestinal infections (Alvarado-Martinez et al., 2020; De Paula Menezes Barbosa et al., 2020; Gato et al., 2020; Moral-Anter et al., 2020). *Lactococcus lactis* subsp. *lactis* CECT 185 was selected as a potential probiotic strain in intestinal environments (Moreno-Chamba et al., 2022). For interactions with HaCaT cells, *Escherichia coli* CECT 515 and *Staphylococcus aureus* CECT 59 were chosen because of their relevance to skin infections and common skin contaminations (Fournière et al., 2020; Friedman et al., 2013; Peres et al., 2015; Tsuru et al., 2021). Additionally, *Cutibacterium acnes* CECT 5684 was included as a representative commensal bacterium in the skin environment (Spittaels et al., 2020; Tsuru et al., 2021). These strains were selected as representatives to study both pathogenic and beneficial bacteria interaction with cell hosts in intestinal and skin environments. Prior each assay, fresh cultures were obtained by culturing each strain in their specific media and conditions (Supplementary material Table S5). The optical density measured at 600 nm (OD_{600}) was used to normalize the concentration of all bacterial suspensions prior each assay to 0.5 ($\sim 10^8$ CFU/ml) (Moreno-Chamba et al., 2023), using a microplate reader.

Cytotoxic effect of polyphenolic-rich extracts in human and bacterial cells

Assessment of antibacterial activity

Minimum inhibitory concentration (MIC) of extracts were determined using the broth microdilution method following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2018). Extracts were two-fold serially diluted (15.00 to 0.03 mg/ml) in Mueller-Hinton medium and 1:1 mixed with fresh bacterial suspensions (OD_{600}=0.5) to reach 200 µl per well. Abiotic control (culture medium with extract) and negative control (culture medium with bacteria) were included. Kanamycin, gentamycin and erythromycin (25.00 to 0.05, 10.00 to 0.02 and 2.00 to 0.004 µg/ml, respectively) were included as positive controls. After 24 h at 37 °C, the inhibition at each dose was measured the OD_{600} and calculated according to negative control. The MIC was confirmed by adding 10 µl of 0.5 % of 2,3,5-triphenyl tetrazolium chloride solution in each well. Wells without red coloration after 30 min at 37 °C were visually identified as the MIC, while successive wells as the SICs for further assays.

Human cells viability

Caco-2 and HaCaT cells were seeded at a density of 1.5×10^4 cells/ml in 96-well plates, incubated and allowed to progress through mature stages of differentiation (8-day or 2-day models, respectively). Cells were incubated for 24 h with two-fold serially diluted polyphenolic extracts (15.00 to 0.03 mg/ml) in antibiotic-free Dulbecco's modified eagle medium (DMEM). Untreated cells were included as control. The viability of cells was estimated by examining their metabolic activity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or adhesion capacity by crystal violet (CV) staining (Gea-Botella et al., 2021; Salazar-Bermeo et al., 2021), using a microplate reader. The viability was normalized as percentage of viability of the control (untreated cells).

Co-culture of bacteria and human cells exposed to polyphenolic-rich extracts

Anti-adhesive effect of polyphenolic-rich extracts in bacteria-challenged cells

For adhesion, human cells (1×10^5 cells/ml) were plated in a 24-well plate to assess a 4-day model of HaCaT or 15-day model of Caco-2 cells. Culture medium was replaced with antibiotic-free DMEM supplemented with SICs of polyphenolic extracts (1/2 × MIC or 1/4 × MIC), corresponding to their effect against each pathogen. Simultaneously, 10^3 CFU/ml of pathogenic bacteria were added to each well and co-incubated for 2 h (37 °C at 5 % CO_2). *K. pneumoniae* and *S. enterica* were used to challenge Caco-2 cells, while *E. coli* and *S. aureus* were used against HaCaT cells. Bacteria-challenged cells with antibiotic-free DMEM (negative) or with p/s-DMEM (positive) were used as controls. Cells with antibiotic-free DMEM without bacteria were included as blanks (sterility) for the assay. After incubation, planktonic cells were washed out three times with 1 × PBS (phosphate buffered saline) and trypsinized with trypsin-ethylenediaminetetraacetic acid. An aliquot of 100 µl of trypsinized suspension of each well was plated in Luria-Bertani (LB) agar plates. Bacterial adhesion was normalized to count of negative control as percentage of adhered bacterial to Caco-2 or HaCaT cells (Gea-Botella et al., 2021; Xu et al., 2015).

Adhesion assay observation

Effect of *S. enterica* on untreated and PePE-treated Caco-2 cell monolayers were replicated on coverslips in 6-well plate and imaged by inverted microscope and field emission scanning electron microscope (FESEM). Cells were treated for 30 min with 4 % glutaraldehyde, washed out three times with 1 × PBS and progressively dehydrated with ethanol (30, 50, 70, 80, 90, and 100 % for 15 min each) (Moreno-Chamba et al., 2023). Micrographs of damaged monolayers were recorded by Sigma 300 VP FESEM (Carl Zeiss, Oberkochen, Germany) at 20 kV without coating.

Anti-invasive effect of polyphenolic-rich extracts in S. enterica-challenged Caco-2 cells

In this assay (Birhanu et al., 2021; Xu et al., 2015), Caco-2 cells were plated as described in the adhesion assay to obtain a 15-day model. Then, the culture medium was replaced with antibiotic-free DMEM supplemented with SICs (1/2 × MIC and 1/4 × MIC) of extracts. Simultaneously, 10^3 CFU/ml of *S. enterica* was added to each well to assess its invasion mechanism (Birhanu et al., 2021; Li et al., 2014). The plates were centrifuged for 45 s and incubated for 2 h at 37 °C under 5 % CO_2. Bacteria-challenged cells with antibiotic-free DMEM (negative) or with p/s-DMEM (positive) were used as controls. After incubation, the cells were washed twice with 1 × PBS and further incubated with DMEM supplemented with gentamycin (20 µg/ml) for 1 h. After killing extracellular bacteria, the cells were washed twice with 1 × PBS and lysed with cold 0.1 % Triton X-100. The lysates from each well were sonicated, diluted, and plated on LB agar. The plates were further incubated at 37 °C for 24 h. After incubation, bacterial count was normalized to the negative control as percentage as bacterial invasion to Caco-2 cells.

Binding site occupation stimuli by polyphenolic-rich extracts in pathogenic/non-pathogenic bacteria interaction

The effect of polyphenolic extracts to stimulate non-pathogenic bacteria to occupy binding sites in human cell monolayers instead of pathogens was assessed according to Jeng and Yan (2022). Caco-2 and HaCaT cells were seeded in 24-well plates as previously stated and incubated with polyphenolic extracts (1/2 × MIC of pathogenic bacteria) diluted in antibiotic-free DMEM. *K. pneumoniae* or *S. enterica* were incubated alongside *L. lactis*, a non-pathogenic bacterium of human intestine (De Paula Menezes Barbosa et al., 2020). *S. aureus* or *E. coli* were incubated along with *C. acnes*, a prevalent bacterium of human skin microbiome (Spittaels et al., 2020).

Three assays were performed simultaneously. A competitiveness assay was carried out, where 10^3 CFU/ml of both potential competitors were added to human cells, followed by incubation for 2 h at 37 °C under 5 % CO_2. For the exclusion assay, the non-pathogenic bacteria were added first to the cell monolayers, and after 1 h, the pathogenic bacteria were added and further incubated for 1 h. For the displacement assay, the same protocol was carried out, but the pathogenic bacteria were added first. For all experiments, planktonic cells were washed out three times with 1 × PBS and trypsinized with 200 µl of trypsin-EDTA. Bacterial counts were carried out on corresponding selective culture media: McConkey agar for *E. coli* and *K. pneumoniae,* Xylose Lysine Deoxycarboxylase agar for *S. enterica*, Mannitol salt agar for *S. aureus*, Man, Rogosa and Sharpe agar for *L. lactis*, and Reinforced Clostridia Medium for *C. acnes*. Solely responsible effect of non-pathogenic bacteria against the pathogenic ones was used as control. All counting data was normalized to the number of adhered pathogenic bacteria to monolayers.

Effect of polyphenolic-rich extracts in lipopolysaccharides (LPS)-challenged human cells

Transepithelial electrical resistance (TEER) determination in human cell monolayers

The integrity of the monolayer formed by Caco-2 or HaCaT cells exposed to LPS was assessed (Chen et al., 2017). Briefly, 1×10^5 cells were seeded in 0.4 µm inserts and incubated in 6-well plates until 21 days (Caco-2) or 8 days (HaCaT). Monolayers with TEER values > 400 Ω cm^2 were used for the LPS-challenge test. LPS from *E. coli* O111:B4 was added apically to Caco-2 and HaCaT cells (25 or 50 µg/ml of LPS, respectively). To weaken cell monolayers to LPS effect, 50 ng/ml of tumour factor necrosis alpha (TNF-α) were also added in the basolateral side of each insert. Simultaneously, polyphenolic extracts at 1/2 × MIC or 1/4 × MIC against *E. coli* were added apically and co-incubated with LPS for 48 h with antibiotic-free DMEM. TEER values were recorded at 0, 1, 2, 4, 6, 24, and 48 h during incubation using a Millicell-Electrical Resistance System (Ers-2) volt-ohm meter. LPS-challenged cells were included as negative control. All data was normalized to TEER values of non-challenged and untreated cells.

Detection of pro-inflammatory interleukins by enzyme linked immunosorbent assay (ELISA)

Supernatants of the cells exposed to LPS, from TEER determination assay, were used to assess the levels of interleukin 6 (IL-6) and interleukin 8 (IL-8) proteins released by the cells using commercial Human Diaclone ELISA kit (Diaclone SAS, Besançon, France).

Evaluation of intracellular ROS generation

Intracellular ROS levels were detected by 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe (Gea-Botella et al., 2021). Caco-2 or HaCaT cells (1.5×10^4 cells/ml) were seeded into 96-well black plates. An 8-day model of Caco-2 and a 2-day model of HaCaT cells were challenged with LPS and treated with polyphenolic extracts (1/2 × MIC and 1/4 × MIC) for 48 h, as stated previously. Then, cells were washed out and treated with 25 µM H_2DCFDA for 40 min at 37 °C. Cells were washed three times with 1 × PBS and then their fluorescence was detected at 490/520 nm of excitation/emission in a microplate reader. Representative micrographs were captured. LPS-challenged but non-extract treated cells (negative control) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)-treated cells (positive control) were included. The results were normalized to negative control.

Mode of cytoprotective action of polyphenolic-rich extracts

Nrf2 CRISPR/Cas9 knockdown (KD)

For the generation of the Nrf2 KD cells, Caco-2 cells were co-transfected with Nrf2-specific CRISPR/Cas9 knockout (KO) plasmid and homology-directed repair (HDR) plasmid using the UltraCruz® Transfection reagent, following manufacturer instructions (Santa Cruz Biotechnology, Inc.) (Ordonez et al., 2022). Co-transfection efficiency was confirmed with fluorescence microscopy by detecting cells emitting both green fluorescence protein (Nrf2 CRISPR/Cas9 KO plasmid) and red fluorescence protein (Nrf2 HDR plasmid) (Supplementary material Fig. S1). Co-transfected cells were selected for 2 weeks with media containing puromycin (4 µg/ml) prior assays.

Determination of cytoprotective effect of polyphenolic-rich extracts in Nrf2 KD cells

After obtaining the KD model, the levels of IL-6, IL-8, and ROS generation were determined in Nrf2 KD Caco-2 cells along with non-transfected Caco-2 cells (control). The cells were challenged with LPS as stated previously along with polyphenolic extracts (1/2 × MIC and 1/4 × MIC) for 48 h at 37 °C under 5 % CO_2. After incubation, the supernatants of each treatment were recovered and stored for IL-6 and IL-8 determinations. Simultaneously, cells were washed out with 1 × PBS, probed with 25 µM of H_2DCFDA, and further incubated for ROS determination as mentioned previously. Additionally, the cells were challenged with *S. enterica* to replicate the invasion assay as stated previously.

In silico docking of Nrf2-Keap1 with the main polyphenolic compounds found in samples

The dimensional structure of Nrf2 was downloaded from PDb (7eca). Structures of the main polyphenolic compounds found in GrPE, PePE, and PoPE were obtained with PubChem in SMILES code. Unsupervised molecular docking was performed in SwissDock AutoDock Vina web-based server to find out the interactions of polyphenols with the conserved motif or active site residues of Nrf2-Keap1 complex. The ligand and target molecules were prepared, and the search was defined using the same grid-box-based coordinates for the Nrf2-Keap1 complex. The binding affinity (kcal/mol) of polyphenols with Nrf2 were analyzed and their interaction was visualized using the online SwissDock AutoDock Vina version.

Statistical analysis

All the determinations in the assays used were performed in triplicate ($n = 3$) independently and expressed as mean ± standard deviation. GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA) was used to perform the statistical analysis. One- or two-way analysis of variance (ANOVA) with Tukey's post hoc test for polyphenolic characterization and antioxidant activity, Dunnett's post hoc test for cellular determinations, and Bonferroni's post hoc test to determine differences between Nrf2 KD and non-KD cells, with p values less than 0.05 ($p < 0.05$), indicating significance. A principal component analysis (PCA) and a Pearson's correlation was performed between TPC, TFC, antioxidant activity of extracts and their biological effects using PAST (PAleontological STatistics) 4.12b statistical software. A PCA between the polyphenolic profile of the extracts and their effect in Nrf2 KD cells was also performed.

Results

Composition of polyphenolic-rich extracts

The PoPE had the highest TPC (403.47 ± 16.53 mg GAE/g) (Supplementary material Fig. S2A) and TFC (254.75 ± 22.43 mg QE/g) (Supplementary material Fig. S2B) ($p < 0.0001$), followed by the GrPE with TPC and TFC values of 145.58 ± 5.72 mg GAE/g and 101.53 ± 11.49 mg QE/g respectively, while PePE had comparatively lower contents. The PoPE antioxidant activity determined by DPPH (1530.63 ± 162.47 mg TE/g; $p < 0.01$, Supplementary material Fig. S2C) and ABTS (13216 ± 563.8 mg TE/g; $p < 0.0001$, Supplementary material Fig. S2D) assays mirrored its TPC, followed by the GrPE and PePE antioxidant activities.

Overall, 72 compounds were identified in the polyphenolic-rich extracts using LC-DAD-MS/MS analysis (Table 1 and Supplementary material Fig. S3). In the GrPE, 64 compounds were identified, primarily composed of flavonoids, especially flavonols and anthocyanins., while phenolic acids were also present. The anthocyanins were the predominant compounds in this sample, such as delphinidin 3-*O*-glucoside, malvidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside, accounting for 39.30 ± 1.61, 16.88 ± 0.56, and 13.29 ± 0.90 mg/g of dry sample, respectively. Sinapic acid was also significant, with a concentration of 26.81 ± 2.16 mg/g of dry sample. In the PoPE, 59 compounds were identified, predominantly phenolic acids (e.g., ellagic acid at 38.71 ± 1.17 mg/g) and flavonoids (e.g., cyanidin 3-o-glucoside at 28.05 ± 2.14 mg/g). The ellagitannins punicalagin-α and punicalagin-β were the major constituents, with concentrations of 25.85 ± 1.57 mg/g and 162.61 ± 4.94 mg/g of dry sample, respectively. PePE exhibited the least diversity, with 42 compounds identified. It was mainly composed of phenolic acids, being gallic acid the most abundant compound at 36.65 ± 2.40 mg/g of dry sample, and protocatechuic acid hexoside at 2.12 ± 0.06 mg/g. In the PePE, low anthocyanin content was also found, being malvidin 3-*O*-glucoside the predominant at 6.19 ± 0.35 mg/g of dry sample.

These results were obtained taking into account Parejo et al. (2004) who developed and validated an HPLC method for analyzing antioxidative phenolic compounds in fennel, highlighting the significance of narrow bore reversed-phase columns for precise separation, and Rajauria (2018) who optimized and validated an RP-HPLC method for polyphenol assessment in seaweed, showcasing the method's robustness and precision.

Toxicity

Antibacterial activity

The antibacterial potential of PePE, GrPE, and PoPE was determined against a panel of bacterial strains (Supplementary material Table S6). PePE exhibited the most pronounced inhibitory effects across all tested bacteria at the lowest polyphenolic concentrations, surpassing the inhibitory activities of GrPE and PoPE. Specifically, PePE showed the lowest MIC against *E. coli* at 0.23 mg/ml of extract (corresponding to 0.01 mg/ml phenolic content), while its highest MIC was observed against *C. acnes* at 7.50 mg/ml of extract (corresponding to 0.37 mg/ml phenolic content). The GrPE displayed the highest reduction in the population of *E. coli* at 0.47 mg/ml (with 0.07 mg/ml phenolic content), whereas its highest MIC (for 3.75 mg/ml of extract with 0.54 mg/ml phenolic content) was observed against *L. lactis, S. aureus*, and *C. acnes*. There were necessary higher concentrations of PoPE to inhibit any of the bacterial species tested, particularly inhibitory effects against *K. pneumoniae* and *L. lactis* were observed at 7.50 mg/ml of extract (with 3.00 mg/ml phenolic content); its lowest MIC was recorded against *S. aureus* (for 0.23 mg/ml of extract with 0.09 mg/ml phenolic content). Consequently, SICs of different extracts against pathogenic bacteria were identified, ensuring the preservation of non-pathogenic bacterial viability for subsequent assays. MICs of antibiotics (kanamycin,

Table 1
Profile of compounds (mg/g of dry sample) found in polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE).

N°	Compound	GrPE	PePE	PoPE
1	Gallic acid*	5.06 ± 0.27	36.65 ± 2.40	9.98 ± 0.30
2	Vanillic acid derivative	< LOQ	< LOQ	< LOQ
3	Coumaryl acid hexoside 2	6.10 ± 0.16	1.26 ± 0.10	0.43 ± 0.02
4	(epi)Gallocatechin pelargonidin hexoside	< LOQ	N.D.	1.50 ± 0.07
5	Protocatechuic acid hexoside	0.12 ± 0.00	2.12 ± 0.06	N.D.
6	Delphinidin 3,5-*O*-diglucoside	0.27 ± 0.02	< LOQ	< LOQ
7	2-*O*-galloylpunicalin	N.D.	N.D.	< LOQ
8	Galloyl glucoside	0.08 ± 0.01	< LOQ	0.05 ± 0.00
9	Digalloyl HHDP hexoside	< LOQ	< LOQ	< LOQ
10	Pelargonidin 3,5-*O*-diglucoside	< LOQ	< LOQ	0.08 ± 0.00
11	Cyanidin 3,5-diglucoside	0.06 ± 0.00	< LOQ	2.72 ± 0.14
12	Vanillic acid hexoside	0.02 ± 0.00	< LOQ	< LOQ
13	Punicalagin-α*	N.D.	N.D.	25.85 ± 1.57
14	Dehydro-galloyl HHDP hexoside	< LOQ	N.D.	< LOQ
15	Protocatechuic acid*	0.64 ± 0.03	0.11 ± 0.00	< LOQ
16	Brevifolin carboxylic acid	0.02 ± 0.00	< LOQ	0.07 ± 0.00
17	Punicalagin-β*	N.D.	N.D.	162.61 ± 4.94
18	Galloyl HHDP glucoside	< LOQ	< LOQ	< LOQ
19	Caffeic acid hexoside	< LOQ	< LOQ	< LOQ
20	Ellagic acid derivative	< LOQ	< LOQ	0.06 ± 0.00
21	Ellagic acid derivative 2	< LOQ	N.D.	0.07 ± 0.01
22	Punigluconin	N.D.	N.D.	< LOQ
23	Coumaryl acid hexoside	0.68 ± 0.04	< LOQ	0.53 ± 0.03
24	Galloyl Bis HHDP hexoside	N.D.	N.D.	< LOQ
25	Rutin	< LOQ	N.D.	N.D.
26	Catechin*	< LOQ	< LOQ	2.78 ± 0.14
27	Epicatechin*	< LOQ	< LOQ	4.47 ± 0.14
28	Chlorogenic acid*	0.67 ± 0.06	0.76 ± 0.05	< LOQ
29	Hydroxybenzoic acid hexoside	< LOQ	N.D.	< LOQ
30	Ferulic acid hexoside derivative	< LOQ	N.D.	N.D.
31	Protocatechuic acid pentoside	< LOQ	< LOQ	N.D.
32	Delphinidin 3-*O*-glucoside	39.30 ± 1.61	1.11 ± 0.07	6.75 ± 0.20
33	Bis HHDP glucoside	< LOQ	< LOQ	< LOQ
34	Malvidin-3-glucoside*	16.88 ± 0.56	6.19 ± 0.35	1.01 ± 0.03
35	Phloretin hexoside	< LOQ	N.D.	N.D.
36	Eriodyctiol hexoside	< LOQ	N.D.	N.D.
37	Vanillic acid*	< LOQ	< LOQ	N.D.
38	Kaempferol couomaroyl glucoside	< LOQ	N.D.	< LOQ
39	Digalloyl glucoside	< LOQ	N.D.	< LOQ
40	Trisgalloyl HHDP hexoside	< LOQ	< LOQ	< LOQ
41	Kaempferol glucoside	< LOQ	N.D.	< LOQ
42	Ferulic acid hexoside	< LOQ	N.D.	< LOQ
43	Syringetin hexoside	< LOQ	N.D.	N.D.
44	Proanthocyanidin B2	< LOQ	N.D.	< LOQ
45	Ellagic acid pentoside	< LOQ	N.D.	0.01 ± 0.00
46	Eschweilenol C	0.02 ± 0.00	< LOQ	0.73 ± 0.05
47	Ellagic acid hexoside	0.21 ± 0.01	0.03 ± 0.03	1.68 ± 0.13
48	Ellagic acid*	0.05 ± 0.00	< LOQ	38.71 ± 1.17
49	Myricetin rhamnoside	< LOQ	N.D.	N.D.
50	Pelargonidin 3-o-glucoside	0.19 ± 0.01	0.19 ± 0.14	< LOQ
51	Cyanidin rutinoside	5.82 ± 0.18	0.07 ± 0.00	9.08 ± 0.14

(*continued on next page*)

Table 1 (*continued*)

N°	Compound	GrPE	PePE	PoPE
52	Cyanidin 3-o-glucoside	13.29 ± 0.90	1.22 ± 0.06	28.05 ± 2.14
53	Ferulic acid	< LOQ	N.D.	< LOQ
54	Quercetin coumaryl glucoside	< LOQ	N.D.	N.D.
55	Myricetin	0.02 ± 0.00	N.D.	< LOQ
56	Cyanidin pentoside	0.24 ± 0.02	0.70 ± 0.05	0.68 ± 0.02
57	Myricetin glucoside	0.06 ± 0.00	N.D.	< LOQ
58	*trans*-Cinnamic acid*	N.D.	< LOQ	N.D.
59	Quercetin*	0.12 ± 0.01	< LOQ	< LOQ
60	Kaempferol rutinoside	< LOQ	N.D.	N.D.
61	Apigenin	N.D.	N.D.	< LOQ
62	Kaempferol*	< LOQ	< LOQ	< LOQ
63	Sinapic acid*	26.81 ± 2.16	0.01 ± 0.00	2.71 ± 0.08
64	Pelargonidin pentoside hexoside	< LOQ	< LOQ	< LOQ
65	Cyanidin pentoside hexoside	1.53 ± 0.09	0.29 ± 0.01	0.05 ± 0.00
66	Syringetin	< LOQ	N.D.	0.12 ± 0.00
67	Valoneic acid bilactone	< LOQ	< LOQ	2.72 ± 0.15
68	Caffeic acid*	0.02 ± 0.00	< LOQ	0.15 ± 0.01
69	ρ-Coumaric acid*	10.67 ± 0.90	< LOQ	7.40 ± 0.22
70	Chlorogenic acid glucoside	0.16 ± 0.00	N.D.	N.D.
71	Punicalin	N.D.	N.D.	< LOQ
72	Galloyl HHDP gluconate isomer	< LOQ	< LOQ	< LOQ

* Compounds were identified with pure standards. LOQ: limit of quantification. N.D.: non detected. Data is expressed as mean ($n = 3$) ± standard deviation.

gentamycin and erythromycin) were also determined (Supplementary material Table S7). Results suggested that at MICs, antibiotics did not cause a higher inhibition of population than polyphenolic extracts, moreover, no selectivity between bacterial strains was observed.

Viability of human cells

CV and MTT assays were employed to assess the impact of extracts on the viability of Caco-2 and HaCaT cells. Results obtained from Caco-2 cells indicated the extracts did not induce cytotoxic effects below 0.94 mg/ml by CV staining (Supplementary material Fig. S4A) and 7.50 mg/ml by MTT assay (Supplementary material Fig. S4B). In CV staining, PePE (1.88 to 7.50 mg/ml of extract) exhibited a modest reduction in Caco-2 cell viability ($p < 0.05$), while GrPE and PoPE reduced viability of these cells between 3.75 to 7.50 mg/ml ($p < 0.01$). In MTT assay, no loss of Caco-2 cell metabolic activity was observed at the extract concentration range 0.03 to 3.75 mg/ml; however, at 7.50 mg/ml, all extracts reduced about 10 % of cell viability ($p < 0.05$). Viability of HaCaT cells was reduced by PePE at 15.00 mg/ml ($p < 0.05$) in CV staining, while PoPE reduced in more than 10 % cell viability at 3.75 mg/ml ($p < 0.01$), followed by GrPE at 0.94 mg/ml ($p < 0.01$). Metabolic activity of HaCaT cells was also reduced by PePE at 3.75 mg/ml ($p < 0.05$), followed by GrPE and PoPE at 0.94 mg/ml ($p < 0.05$).

These findings suggested that the three extracts did not exhibit toxic effects on both human cell lines at doses corresponding to bacterial SICs; thus, these extracts can be applied in co-culture experiments without compromising human cell viability.

Anti-adhesion assay

The anti-adhesion effect of extracts was assessed in Caco-2 and HaCaT cells. Extract SICs (1/2 × MIC and 1/4 × MIC) were used to not interfere with bacterial and human cell viability. Results showed that extracts reduced the adhesion of the pathogenic bacteria used to Caco-2 cells after 2 h of coincubation (Fig. 1A). At 1/2 × MIC, GrPE and PePE showed substantial reduction of *S. enterica* adhesion of 70 and 80 %, respectively. The effect of PePE surpassed the effect of the positive control ($p < 0.01$). Additionally, both PePE and PoPE exhibited 60 % reduction of *K. pneumoniae* adhesion, an effect comparable to the positive control ($p > 0.05$). This reduction was consistent even at 1/4 × MIC by all samples. Microscopic examination (Fig. 1C and Supplementary material Fig. S5) provided further insights, revealing that *S. enterica* induced a loss of Caco-2 cell monolayer integrity whereas treatment with phenolic extracts preserved its integrity during the assessment.

Upon challenging HaCaT cells with *S. aureus* and *E. coli* (Fig. 1B), all phenolic extracts consistently reduced the percentage of adhered bacterial cells ($p < 0.0001$). At 1/2 × MIC, GrPE, PePE and PoPE diminished *S. aureus* adhesion by 50 %, 60 %, and 70 % respectively, being last reduction similar to the positive control ($p > 0.05$). Furthermore, all extracts exhibited a reduction over 30 % of *E. coli* adhesion with PePE matching the effect of the positive control ($p > 0.05$). GrPE at 1/4 × MIC caused significantly less reduction in *E. coli* adhesion to HaCaT cells than the positive control ($p < 0.01$).

Anti-invasion assay

The ability of polyphenolic-rich extracts to reduce the invasive mechanisms of *S. enterica* was evaluated (Fig. 2). Overall, all extract samples reduced *S. enterica* invasion over 60 % ($p < 0.001$) with PoPE matching comparable to positive control ($p > 0.05$). Noteworthy, even at 1/4 × MIC, the invasion of *S. enterica* was reduced in more than 30 % ($p < 0.01$) by all samples.

Binding site occupation stimuli by polyphenolic-rich extracts in pathogenic/non-pathogenic bacteria interaction

Given the observed potential of polyphenolic extracts to diminish the adhesion of pathogenic bacteria to human cells, the potential extract-derived stimuli that could promote the occupation of cell attachment sites by non-pathogenic versus pathogenic bacteria were also determined. *L. lactis* was able to prevail and adhere to Caco-2 cells, excluding both *S. enterica* and *K. pneumoniae* over 40 % (Fig. 3A). Bacterial strain exhibited a modest yet discernible effect in displacing both pathogens by more than 20 %. When human cells were treated with 1/2 × MIC of any extract, a noted reduction over 60 % ($p < 0.001$) and 70 % ($p < 0.01$) in the cell adhesion of *S. enterica* and *K. pneumoniae* respectively was observed. Thus, the competitiveness of *L. lactis* was enhanced. GrPE and PePE promoted the exclusion of *S. enterica* and *K. pneumoniae* by *L. lactis* ($p < 0.01$), while PoPE showed no effect against *K. pneumoniae* ($p > 0.05$). In displacement assays, phenolic extracts amplified the effect of *L. lactis*, especially PePE ($p < 0.001$) and GrPE ($p < 0.0001$).

In HaCaT cells (Fig. 3B), *C. acnes* showed a modest effect in competition against *S. aureus* and *E. coli*, resulting in a 40 % and 30 % reduction in cell adherence, respectively. Moreover, *C. acnes* exhibited a substantial effect in excluding *S. aureus* and *E. coli*, achieving a 70 % and 50 % reduction in cell adherence, respectively. The displacement assay mirrored these effects with *C. acnes* reducing cell adhesion of both pathogens by 40 %. After extract-treatment, *C. acnes* exhibited an improved competitive effect against *S. aureus* and *E. coli*, reducing 70 % and 60 % pathogenic bacterial adhesion, respectively ($p < 0.05$). Also, PePE and PoPE improved the action of *C. acnes* in displacing the adhesion of *S. aureus* and *E. coli* by 60 % ($p < 0.05$), while GrPE showed similar effect to *C. acnes* alone ($p > 0.05$). No improvement was observed in the exclusionary action of *C. acnes* against both bacterial pathogens with the application of extracts ($p > 0.05$).

Cytoprotective effect of polyphenolic-rich extracts in human cells exposed to LPS

The cytoprotective potential of GrPE, PePE and PoPE against LPS-induced impairment of barrier was investigated in Caco-2 and HaCaT

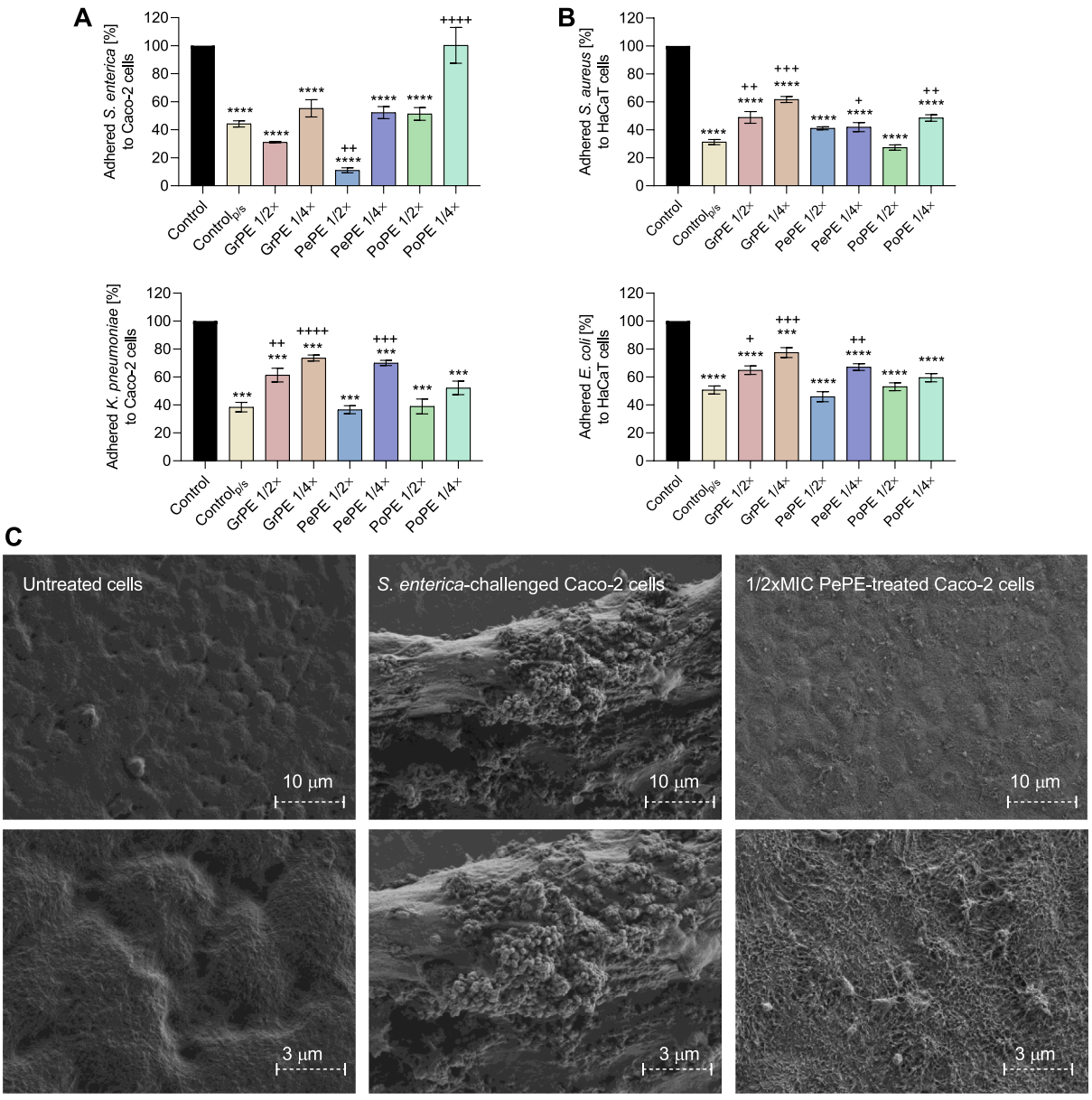

Fig. 1. Polyphenolic extracts of grape (GrPE), pomegranate (PoPE), and persimmon (PePE) reduced pathogenic bacterial adhesion on (A) Caco-2 and (B) HaCaT cell monolayers. The adhesion of pathogens on untreated cells was used as Control (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$; Two-way ANOVA with Dunnett's post hoc test), while a penicillin/streptomycin solution was included as positive control ($Control_{p/s}$) (++++$p < 0.0001$, ++$p < 0.01$; Two-way ANOVA with Dunnett's post hoc test). Data was normalized to the adhesion of pathogens without treatment. Values are expressed as mean ± standard deviation ($n = 3$). (C) Field emission scanning electron microscopy (FESEM) micrographs of untreated, *S. enterica*-challenged, and PePE-treated Caco-2 cell monolayers.

cell monolayers (Fig. 4A). In Caco-2 monolayers, LPS-induced impairment exceeded in 20 % ($p < 0.001$) and persisted throughout the end of incubation. Treatment with polyphenolic-rich extracts, particularly PePE at 1/2 × MIC, exhibited noted protective response, increasing TEER values by 60 % after 24 h when compared to non-challenged and non-treated cells ($p < 0.001$). Monolayers treated with 1/4 × MIC PePE displayed an initial loss in TEER values, recovering to levels comparable to non-challenged and non-treated cells ($p > 0.05$). A reduction in barrier function was noted in Caco-2 cell monolayers treated with 1/2 × MIC GrPE ($p < 0.01$); however, TEER values were promoted by this extract at the end of LPS-exposure similarly to non-challenged cells values ($p > 0.05$). A similar pattern was observed in HaCaT cell monolayers, where LPS induced more than 20 % impairment of barrier ($p < 0.001$). Treatment with PePE, GrPE or PoPE at the same concentration (1/2 × MIC) resulted in comparable TEER values to unchallenged cells ($p > 0.05$). At 1/4 × MIC, PePE and PoPE stimulated TEER values similarly to untreated and unchallenged cells ($p > 0.05$), while GrPE managed to maintain in 10 % the decrease of TEER values at the end of incubation ($p < 0.05$).

Since LPS generates proinflammatory stimulus after cell exposition, the supernatant of cells was recovered to determine anti-inflammatory effects during the interaction of Caco-2 and HaCaT cell monolayers with LPS (Fig. 4B). The treatment with polyphenolic-rich extracts mitigated extracellular production of proinflammatory IL-6 and IL-8 in both Caco-2 and HaCaT cells after 48 h of incubation with LPS. In Caco-2 cell monolayers, 1/2 × MIC treatment of any of the extracts used resulted in lower levels of IL-6 (<50 pg/ml) ($p < 0.01$) and IL-8 (<250 pg/ml) ($p < 0.001$) compared to LPS-challenged cells (>110 pg/ml). The treatments with the PePE and GrPE at 1/2 × MIC reduced with 70 % of the IL-6 ($p < 0.01$) production while the treatment with PoPE reduced IL-8 ($p < 0.01$) production by a similar percentage. Similar patters were observed in HaCaT cells with treatments with PePE and GrPE at 1/2 ×

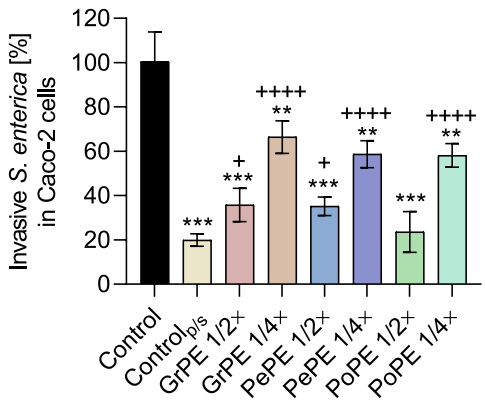

Fig. 2. Polyphenolic extracts of grape (GrPE), persimmon (PePE), and pomegranate (PoPE) reduced *Salmonella enterica* serovar Typhimurium invasion in Caco-2 cell monolayers. Data was normalized and compared to *S. enterica*-challenged cells (Control) (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Two-way ANOVA with Dunnett's post hoc test), while Penicillin/streptomycin was used as positive control ($Control_{p/s}$) (++++$p < 0.0001$, ++$p < 0.01$; Two-way ANOVA with Dunnett's post hoc test). Values are expressed as mean ± standard deviation ($n = 3$).

MIC reducing the IL-6 production by more than 60 % ($p < 0.01$), and PoPE treatment reducing IL-6 production by 50 % ($p < 0.01$). The IL-8 levels in 1/2 × MIC PePE treated HaCaT cells were lower and especially with PoPE ($p < 0.01$), followed by the GrPE ($p < 0.05$) one. These findings confirmed obtained TEER values after 48 h of LPS incubation.

LPS can also cause increase in ROS production in cells as part of the cellular response to combat its effect; thus, ROS production was also measured in the human cells challenged with LPS from *E. coli* O111:B4. After 48 h of incubation, LPS-induced increase in ROS production was effectively mitigated by polyphenolic-rich extracts. At both concentrations (1/2 × MIC and 1/4 × MIC), the extracts reduced ROS production in Caco-2 cells (Fig. 5A) by over 40 % ($p < 0.001$) and with GrPE at 1/4 × MIC exhibiting 20 % reduction ($p < 0.05$). In addition, the PePE ($p < 0.01$) and especially PoPE ($p < 0.001$) treatments, at 1/2 × MIC, showed pronounced reduction in ROS compared to Trolox. In HaCaT cells (Fig. 5B), 1/2 × MIC treatment of any of extracts used reduced by over 20 % ROS production ($p < 0.001$), as well as 1/4 × MIC PoPE treatment ($p < 0.001$). At 1/4 × MIC, PePE and GrPE treatments reduced ROS production by 15 % ($p < 0.01$) and 10 % ($p < 0.05$), respectively. As observed in Caco-2 cells, PePE and PoPE treatments at 1/2 × MIC also showed pronounced anti-ROS effect ($p < 0.001$) in HaCaT cells compared to Trolox. The intracellular antioxidant activity of these extracts further underscored their effects (Fig. 5C).

Effect of polyphenolic-rich extracts on Nrf2 KD Caco-2 cells

To unravel the effect of polyphenolic extracts in the adaptative response of cells, the role of Nrf2 pathway in Caco-2 cell model was

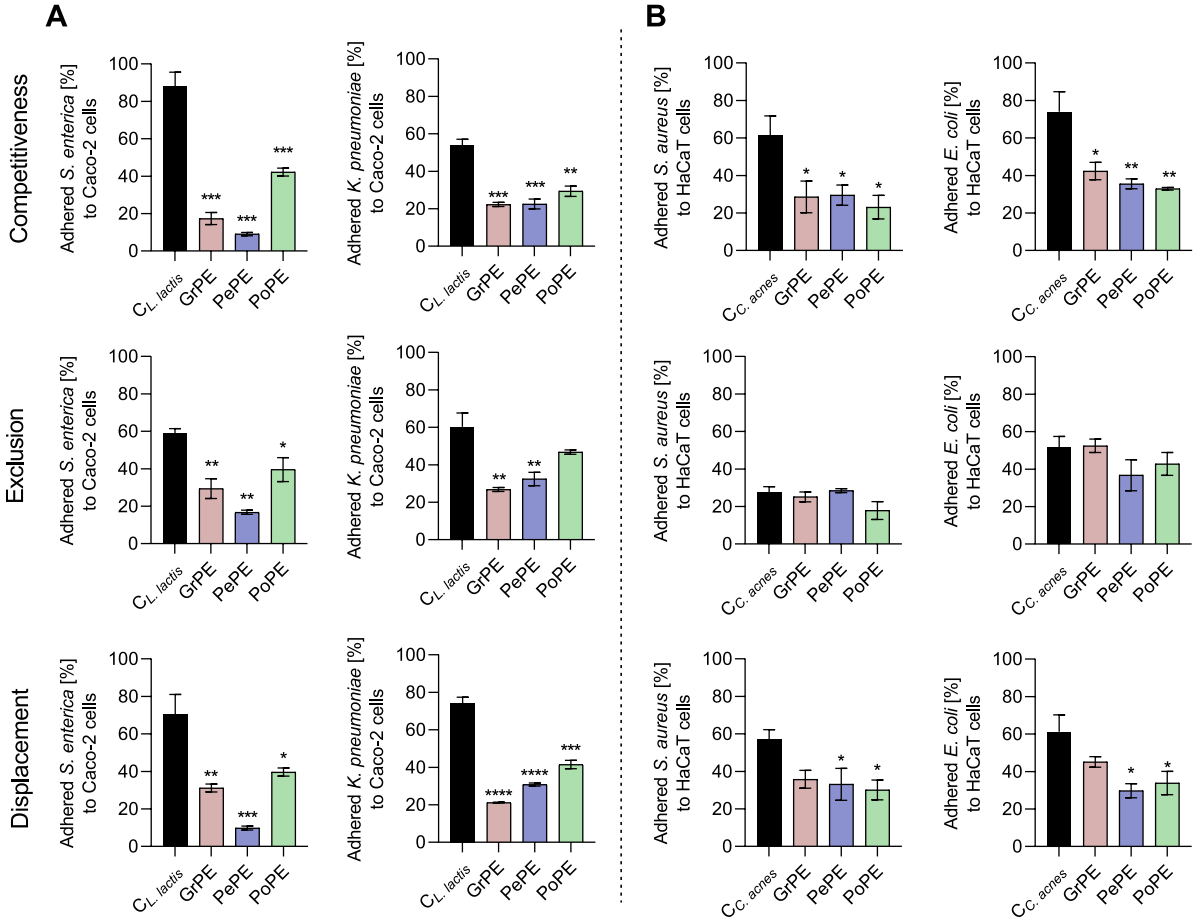

Fig. 3. Polyphenolic extracts of grape (GrPE), pomegranate (PoPE), and persimmon (PePE) modulated the competitiveness, exclusion and displacement interaction of (A) *Lactococcus lactis/Salmonella enterica* or *L. lactis/Klebsiella pneumoniae* and (B) *Cutibacterium acnes/Staphylococcus aureus* or *C. acnes/Escherichia coli*, to occupy binding sites on Caco-2 and HaCaT cells, respectively. The effect of non-pathogenic strains was included as control (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; One-way ANOVA with Dunnett's post hoc test). All data were normalized to the adhered pathogenic strains to untreated human cells. Values were expressed as mean ± standard deviation ($n = 3$).

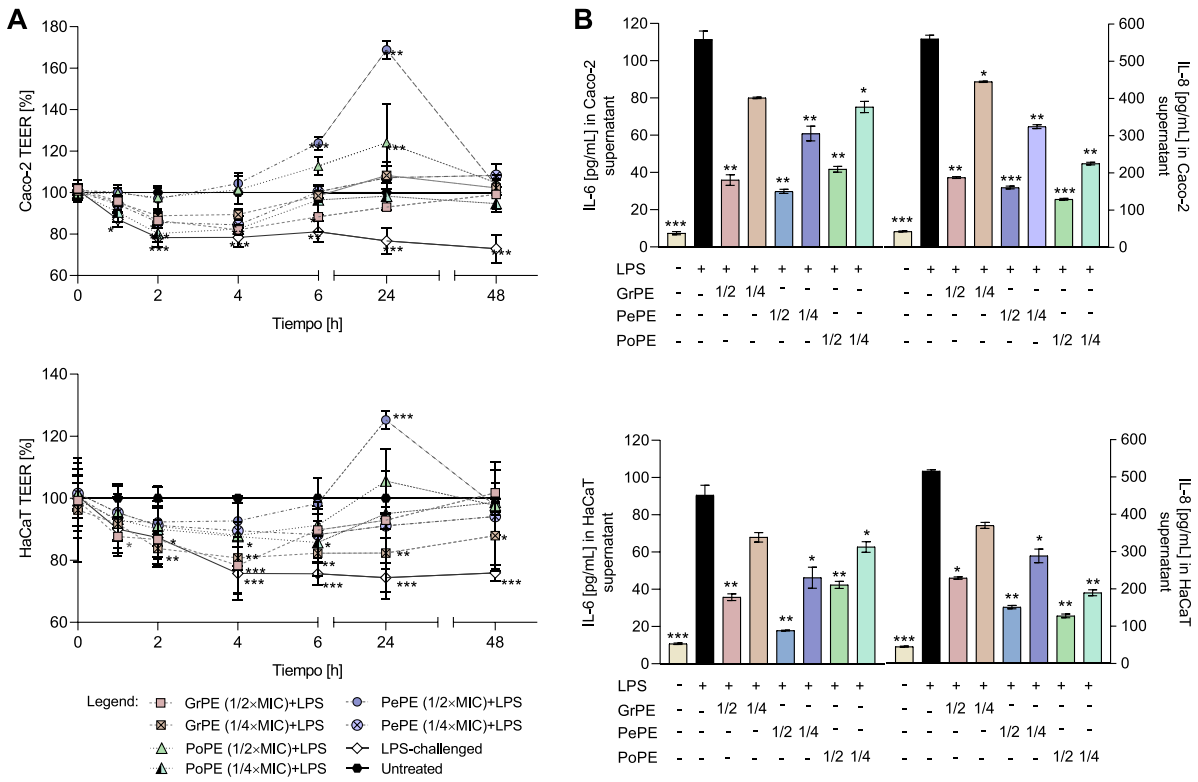

Fig. 4. Polyphenolic extracts of grape (GrPE), pomegranate (PoPE) and persimmon (PePE) showed cytoprotective effects on lipopolysacchardes (LPS) from *E. coli* O111:B4 in Caco-2 and HaCaT cell monolayers during 48 h of incubation. Extracts improved the (A) transepithelial electric resistance (TEER) response of cells during incubation and reduced (B) the levels of pro-inflammatory cytokines interleukin 6 (IL6) and 8 (IL-8) after incubation. Untreated LPS-challenged cells were used as control (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Two-way ANOVA with Dunnett's post hoc test). Data were expressed as mean ± standard deviation ($n = 3$).

investigated. For this Nrf2 KD and non-KD Caco-2 cells were challenged with *S. enterica* in an invasion assay (Fig. 6A). The GrPE and PePE treatments exhibited Nrf2-dependent cytoprotective effect. Interestingly, at both 1/2 × MIC ($p < 0.001$) and 1/4 × MIC ($p < 0.05$), these extracts failed to reduce invasion of *S. enterica* in Nrf2 KD cells in contrast to their effects in non-KD Caco-2 cells. Conversely, the PoPE treatment maintained its protective effect, showcasing consistent reduction of *S. enterica* invasion in both Nrf2 KD and non-KD Caco-2 cells ($p > 0.05$).

When analyzing the anti-inflammatory effects of the extracts against LPS from *E. coli* O111:B4 (Fig. 6B), the results revealed a parallel trend. In Nrf2 KD cells, both PePE and GrPE treatments exhibited loss of their anti-inflammatory effects at both concentrations ($p < 0.001$), while PoPE treatment sustained its effect, reducing the IL-6 and IL-8 levels equivalently in both Nrf2 KD and non-KD Caco-2 cells ($p > 0.05$). Reduction of oxidative stress, as measured by ROS levels, agreed with invasion and interleukins determinations; both GrPE ($p < 0.01$) and PePE ($p < 0.001$) treatments failed to reduce ROS in Nrf2 KD cell model at the tested concentrations, in contrast to their effects in non-KD Caco-2 cells (Fig. 6C and D). Conversely, the PoPE treatment exhibited consistent intracellular ROS reduction in both Caco-2 cell models ($p > 0.05$). These collective findings indicated that at the concentrations tested, the PoPE treatment showcased a cytoprotective mode of action unrelated to Nrf2 pathway stimulation in Caco-2 cells, while the cytoprotective effects of PePE and GrPE were contingent upon Nrf2 pathway activation.

PCA and correlation analysis of samples and their biological activity

A PCA was performed to understand the variability and relationships between different polyphenolic extracts and their biological activities (Supplementary Fig. S6). The analysis indicated that the first principal component (PC1) explained 67.52 % of the variance, while the second principal component (PC2) accounted for 32.44 %. The PCA plot was consistent with previous results, revealing a distinct separation among the extracts: PoPE was positioned positively on PC1, indicating high TPC, TFC, and DPPH and ABTS antioxidant activities. In contrast, PePE and GrPE were situated negatively on PC1, with PePE located in the positive PC2 quadrant and GrPE in the negative PC2 quadrant. PePE showed significant effects on TEER values, IL-6 reduction in cells, and the inhibition of *S. enterica* adherence. GrPE was associated with the exclusion and displacement of *K. pneumoniae*, along with antibacterial activities against *S. aureus, K. pneumoniae*, and *S. enterica*.

These findings showcase the different effects of the extracts depending on the bacterial strain and host response, highlighting their potential for targeted therapeutic applications. A Pearson's correlation analysis was also performed (Supplementary Fig. S7). The results display no obvious correlation between the antibacterial activity of the samples and their antioxidant activity. However, the antioxidant activity of samples, as measured by DPPH, was positively associated with the antibacterial activity of samples against *E. coli* ($p < 0.05$), along with the TPC of samples. Additionally, a negative correlation was observed between TPC and the inhibition of *S. enterica* adhesion in the competitiveness assay ($p < 0.05$). The inhibition of *S. aureus* adhesion in the exclusion assay was positively correlated with the TPC of samples. IL-6 levels in Caco-2 cells were negatively associated with the antioxidant activity of samples, as measured by ABTS ($p < 0.05$).

A PCA between the polyphenolic profile of extracts and their effect in Nrf2 KD Caco-2 cells was also performed (Supplementary Fig. S8). The analysis indicated that PC1 explained 65.13 % of the variance, while PC2 accounted for 34.87 %. The PCA displayed that delphinidin 3-*O*-glucoside, gallic acid, and punicalagin, the major compounds found in GrPE, PePE, and PoPE, influenced the effects observed in Nrf2 KD cells the most.

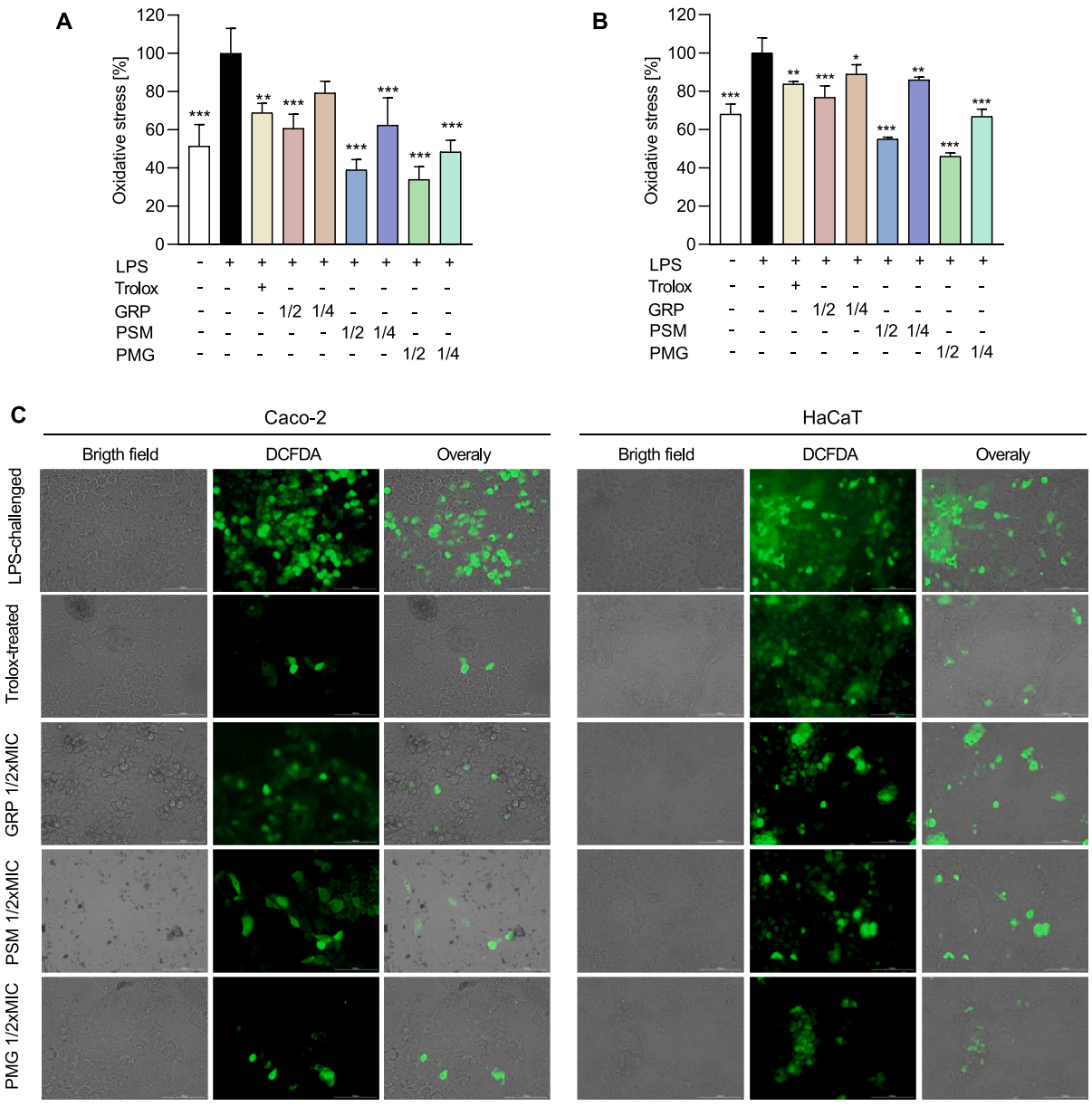

Fig. 5. Intracellular reactive oxygen species (ROS) reduction in (A) Caco-2 and (B) HaCaT cells challenged with lipopolysaccharide (LPS) from *E. coli* O111:B4 by polyphenolic extracts of grape (GrPE), pomegranate (PoPE) and persimmon (PePE). Untreated LPS-challenged cells were included as control (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Two-way ANOVA with Dunnett's post hoc test), along with Trolox, an analog of Vitamin E (+++$p < 0.001$, ++$p < 0.01$, +$p < 0.05$; Two-way ANOVA with Dunnett's post hoc test). Oxidative stress percentage was expressed as mean ± standard deviation ($n = 3$). (C) Representative micrographs of Caco-2 and HaCaT cells labeled with H_2DCFDA, an indicator of reactive oxygen species (ROS).

In silico interactions of polyphenols with Nrf2

Since PCA revealed a strong association between the compounds with the highest concentrations found in GrPE, PePE, and PoPE, respectively, and their effects in Nrf2 KD cells, we analyzed their molecular interactions with the Nrf2-Keap1 complex *in silico* (Fig. 7A). Delphinidin 3-*O*-glucoside in GrPE showed the strongest binding affinity for Nrf2, with energies ranging from –9.345 to –7.681 kcal/mol, indicating robust and stable interactions (Fig. 7B). Gallic acid, the main compound found in PePE, exhibited moderate affinity, with binding energies between –6.771 and –6.303 kcal/mol, suggesting relatively weaker interactions compared to delphinidin 3-*O*-glucoside. Punicalagin, the predominant compound in PoPE, despite showing notable affinity in its top model (–8.030 kcal/mol), displayed significant variability, with energies spanning from –8.030 to –5.429 kcal/mol.

Discussion

The antibacterial potential of polyphenolic extracts from different plant sources has been well-documented by several studies (Alvarado-Martinez et al., 2020; Friedman et al., 2013; Gato et al., 2020; Manso et al., 2021). In this study, the antibacterial potential of GrPE, PePE and PoPE beyond their inhibitory effects in bacterial proliferation was determined, focusing on the protection of cell hosts during interaction with bacteria and their toxins. The work showed that the treatments with GrPE, PePE and PoPE exhibited a strong reduction in the adhesion of the pathogens *S. enterica* and *K. pneumoniae* or *S. aureus* and *E. coli* to Caco-2 and HaCaT cells, respectively.

While anti-adhesive effect of persimmon has been attributed to its carotenoids (Gea-Botella et al., 2021), the impact of its polyphenols had not been explored. Despite its low TPC, PePE exhibited a noted effect by inhibiting some of the tested pathogens with lower MICs, translated in a

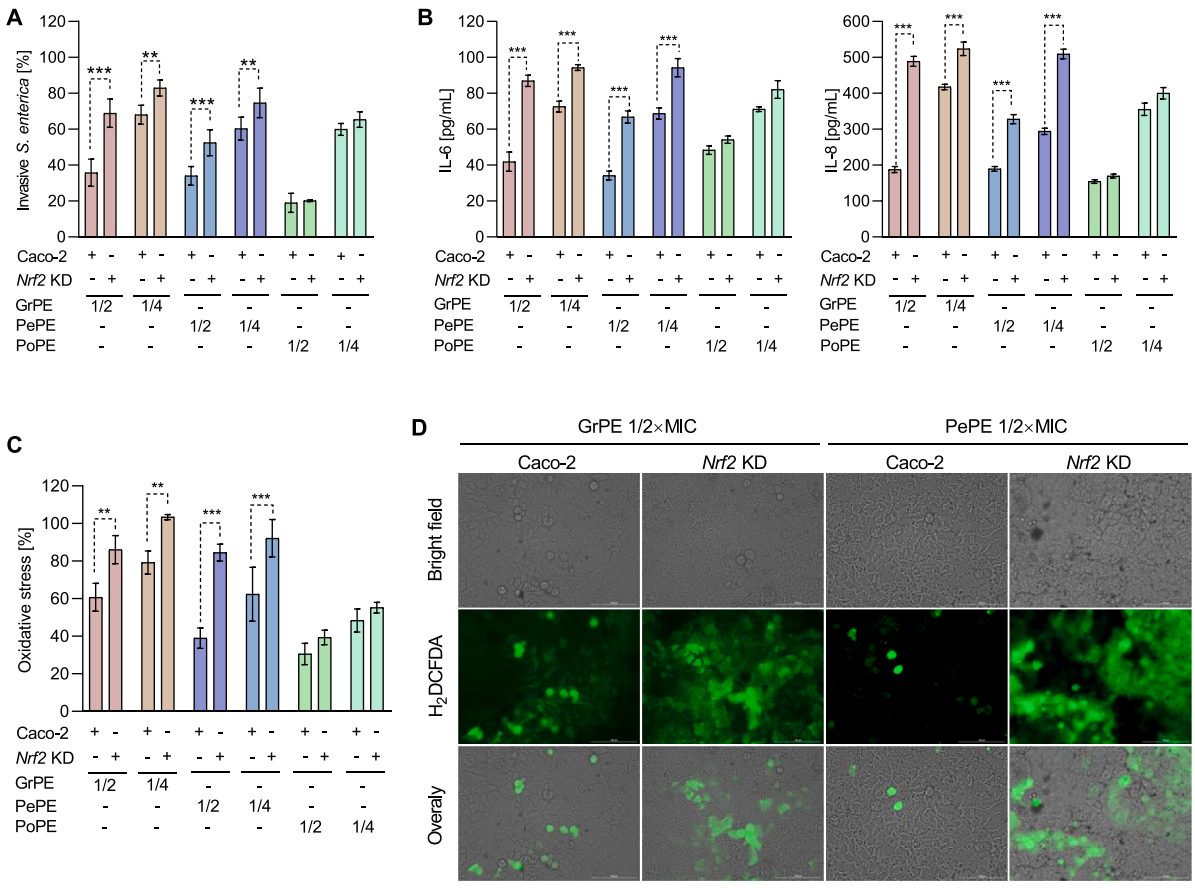

Fig. 6. Effect of polyphenolic extracts of grape (GrPE), persimmon (PePE) and pomegranate (PoPE) in Nrf2 knockdown (KD) Caco-2 cells. (A) Anti-invasive effect of extracts against *Salmonella enterica*. (B) Effect of extracts on interleukin 6 (IL-6) and 8 (IL-8) in cells challenged with lipopolysaccharide (LPS) from *E. coli* O111:B4. (C) Effect of extracts in intracellular reactive oxidative species (ROS) in LPS-challenged Nrf2 KD Caco-2 cells. (D) Representative micrographs of Nrf2 KD Caco-2 cells labeled with H_2DCFDA as indicator of ROS. Non-KD Caco-2 cells were included as control (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Two-way ANOVA with Bonferroni's post hoc test). Data are expressed as mean ± standard deviation ($n = 3$).

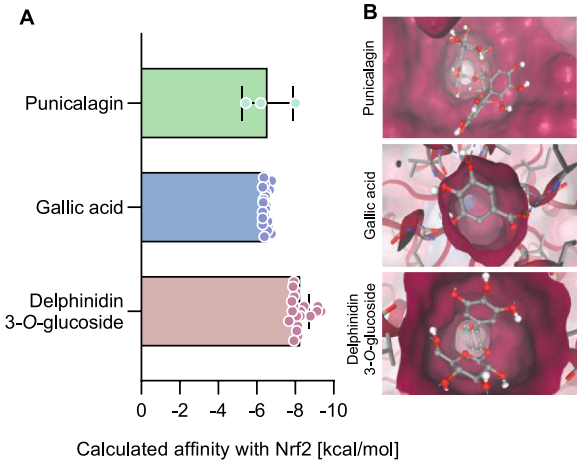

Fig. 7. *In* silico docking interactions of the main polyphenolic compounds found in the polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE) with Nrf2-Keap1 complex. (A) Calculated affinity of punicalagin, gallic acid and delphinidin 3-*O*-glucoside from PoPE, PePE and GrPE, respectively, with Nrf2-Keap1 complex. (B) 3D structure of Nrf2 showing the cavity of affinity with punicalagin, gallic acid, and delphinidin 3-*O*-glucoside.

noted effect against *S. enterica* adhesion on Caco-2 cells, likely due to its high gallic acid content. Gallic acid is known for its pronounced anti-adhesive effect, disrupting the surface free energy of bacterial adhesion (Alvarado-Martinez et al., 2020; Luís et al., 2014; Tanaka et al., 2018). Moreover, PePE, obtained by alkaline and acid hydrolysis, contains bounded polyphenols which are not easily extractable. It has been mentioned that bounded polyphenols may exert a higher activity than available or free polyphenols (Domínguez-Rodríguez et al., 2017; Pérez-Jiménez et al., 2013; Sun et al., 2021), like those found in GrPE and PoPE. This suggest that specific bounded polyphenolic compounds like gallic acid in PePE, could have a noted impact on antibacterial properties due to their reactivity, stability, or conjugation with sugars, potentially enhancing their persistence. Further studies are needed to explore these findings.

Antibacterial activity of punicalagins from pomegranate have been well-documented, especially against Gram-positive bacteria (Li et al., 2014; Silva et al., 2023; Xu et al., 2015), which aligns with the high reduction on *S. aureus* adhesion to HaCaT cells obtained by this sample. However, Its weak anti-adhesive effect reported previously (De Paula Menezes Barbosa et al., 2020; Li et al., 2014) implies that the anti-adhesive effect of PoPE would be due to a synergistic effect from its polyphenolic constituents rather than to its major compounds. GrPE showed also substantial reduction of *S. enterica* adhesion to Caco-2 cells. Grape anthocyanins have exhibited anti-adhesive properties against pathogenic bacteria (Parkar et al., 2014; Silva et al., 2016). In this sense, the delphinidin 3-*O*-glucoside found in GrPE may be associated with its anti-adhesive properties against *S. enterica*.

Pathogenic adhesion correlates with bacterial persistence and

antimicrobial resistance, particularly in biofilm formation. Thus, compounds exhibiting antibiofilm properties may also exert anti-adhesion effects (Silva et al., 2016; Viela et al., 2020; Viljoen et al., 2020). Given that GrPE demonstrated antibiofilm properties in previous research (Moreno-Chamba et al., 2023), a very high anti-adhesive effect was expected. However, the observed effect of GrPE treatment was not as robust. The GrPE anti-adhesive effect could be associated against bacterial adhesins and appendages (Viljoen et al., 2020), consistent with the action of anthocyanins (Parkar et al., 2014) and ellagitannins (Xu et al., 2015), the major compounds of the GrPE and PoPE, respectively. Additionally, the physical disruption of bacterial cell surface by electrostatic and hydrophobic forces impedes pathogenic adhesion to cell hosts (De Paula Menezes Barbosa et al., 2020; Viljoen et al., 2020); therefore, the compounds that target both the bacterial cell membrane and the adhesins may show distinctive anti-adhesive effects. The treatments with PePE and PoPE caused noticeable cell membrane damage while the treatment with GrPE did not exerted that (Moreno-Chamba et al., 2023), which could explain their respective percentage reductions of bacterial adhesion.

Furthermore, the extracts treatments preserved barrier integrity of the cells after the pathogenic adhesion, as observed in the FESEM micrographs. The deleterious effect of *S. enterica* on the monolayer integrity of Caco-2 cells was observed, indicating the invasive nature of this bacterial strain (Birhanu et al., 2021; De Paula Menezes Barbosa et al., 2020; Li et al., 2014). This effect was likely triggered by the production of TNF-α in cells during *S. enterica* interaction (Moral-Anter et al., 2020). The treatments with the extracts, especially with the PoPE, exhibited strong effect against *S. enterica* invasion which can be related to its TFC. Flavonoids have been reported to inhibit bacterial invasion by targeting the virulent type III secretion substrates encoded at the *Salmonella* pathogenicity island 1 locus (Birhanu et al., 2021; Li et al., 2014), showcasing potential intracellular antibacterial activity. Moreover, since polyphenolic-rich extracts may have targeted bacterial adhesins, alterations in adhesins like *S. enterica* type 1 fimbriae (De Paula Menezes Barbosa et al., 2020) might therefore have reduced bacterial invasion.

Polyphenolic compounds also influence non-pathogenic and commensal adhesion to cell hosts, allowing them to prevail against pathogens (De Paula Menezes Barbosa et al., 2020; Parkar et al., 2014; Peres et al., 2015). This selective effect was hinted in the antibacterial assay, where both *L. lactis* and skin-commensal *C. acnes* bacteria required higher doses of phenolic extracts to be inhibited compared to pathogens. This selectivity was further corroborated in the binding site stimuli assay, where polyphenolic extracts supported the effect of both strains to compete for specific cell receptors (competition), block (exclusion), and provoke detachment (displacement) of bacterial pathogens on Caco-2 and HaCaT cells.

L. lactis exhibited a modest *S. enterica* exclusion effect from Caco-2 cells and a moderate competition and exclusion effects against *K. pneumoniae*. The treatments with the GrPE and PePE boosted the effect of this strain, while the treatment with the PoPE induced a modest improvement. This suggests that the interplay between *L. lactis* and polyphenolic-rich extracts translated into a synergistic effect, reducing pathogenic adhesion to Caco-2 cells. Since lactic acid bacteria display β-glucosidase and esterase activity (Peres et al., 2015), *L. lactis* might metabolize certain glycosylated compounds of the extracts, such as anthocyanins in the GrPE, enabling the strain to compete, to exclude, and to displace both pathogens, especially *K. pneumoniae*. The PoPE-derived polyphenolic compounds could have potentially modulated *L. lactis* cell membranes, participating in protein-protein interactions due to their hydroxyl groups (De Paula Menezes Barbosa et al., 2020), thereby enhancing non-pathogenic adhesion to Caco-2 cells. Also, the gallic acid could increase the survival rate and adhesion ability of lactic acid bacteria on the epithelial cells (Chen et al., 2017; Moreno-Chamba et al., 2022), which could also explain the results observed for PePE against *S. enterica*.

C. acnes exhibited modest competition, displacement, and especially exclusion effects against *S. aureus* from HaCaT cells while *E. coli* cells were excluded or displaced. Several studies have reported that *C. acnes* can prevail over pathogens due to its production of propionic acid, bacteriocins (Spittaels et al., 2020), or desensitization of toll-like receptors on HaCaT cells (Fournière et al., 2020). In this context, the treatments with polyphenolic-rich extracts improved the ability of *C. acnes* to compete for binding sites against *S. aureus* and *E. coli*. The PePE and PoPE treatments boosted the displacement effect of *C. acnes*. The exclusion results with polyphenolic treatment might suggest that under tested conditions (SICs and CFUs) the maximum capacity of exclusion of both pathogens was reached. These results showed the potential of the polyphenolic-rich extracts to increase the prevalence of commensal strains over pathogenic bacterial adherence, therefore the capability to promote the reduction of potential infections.

All the three polyphenolic-rich extracts showed activity against bacterial cells when they interact with cell host while they selectively allowed beneficial and commensal strains to prevail on Caco-2 and HaCaT cells. This study results also suggest that the extracts influenced the cellular response mechanisms against bacterial structural virulence factors like LPS, findings which are in agreement with other research (Kim et al., 2023; Silva et al., 2016; Tanaka et al., 2018). This endotoxin plays a key role during the host-Gram-negative pathogen interactions and colonization, compromising cellular function, being translated in overexpression of pro-inflammatory cytokines, in barrier function loss, in the tissue damages and oxidative stress (Kim et al., 2023; Moral-Anter et al., 2020; Viljoen et al., 2020; Xu et al., 2015). In LPS-challenged Caco-2 and HaCaT cells, the treatment with polyphenolic-rich extracts improved their barrier function, reducing the barrier function integrity loss probably by targeting tight junction proteins, tool-like receptors or by stimulating the adaptative cellular response against LPS (Chen et al., 2017; Kim et al., 2023; Tanaka et al., 2018).

The polyphenolic-rich extracts promoted higher TEER values in Caco-2 at early stages of LPS-exposure than in HaCaT cells which required more time to improve them, hinting a faster modulatory effect at intestinal level rather than at the skin level. The inherent composition of each extract might have played a role in this effect. For instance, the PePE and PoPE stimulated the highest TEER values in both cell lines at 24 h of exposure to LPS in a dose-dependent manner, which may be related to the effect of gallic acid and punicalagins in the expression and distribution of the tight junction proteins (Moral-Anter et al., 2020). Interestingly, the treatment with GrPE also improved TEER values in both cells at the end of incubation. Since viability of Caco-2 and HaCaT cells were not compromised at tested doses with extracts, the drop of TEER values could be related to the stimulation of cell membrane permeability rather than exerting cytotoxic effects (Nallathambi et al., 2020). The anthocyanins present in the GrPE can exercise anti-inflammatory effect during the exposure time to LPS (Nallathambi et al., 2020) protecting the cells.

This assumption was verified by the analysis of pro-inflammatory cytokines in cells exposed to LPS. Both IL-6 and IL-8 interleukins are implicated in common processes related to the innate immune system (Kim et al., 2023; Moral-Anter et al., 2020; Romier-Crouzet et al., 2009). When their production surpasses certain threshold, it could lead to tissue damages, barrier function loss or even to the development of the chronic diseases (Ferrari et al., 2016; Kim et al., 2023). The treatment with GrPE managed to reduce both interleukins in Caco-2 and HaCaT cells exposed to LPS, which agrees with the anti-inflammatory effect of anthocyanins against LPS (Ferrari et al., 2016; Gasparrini et al., 2018; Nallathambi et al., 2020). Probably, the reduction of IL-6 and IL-8 by the cyanidin and the delphinidin 3-*O*-glucosides protected tight junction proteins related to closing gaps among adjacent cells, regulating the passage of specific molecules across the epithelium during incubation (Ferrari et al., 2016; Nallathambi et al., 2020). It is worth to mentioning that the IL-6 was particularly affected by the PePE and GrPE treatments while IL-8 was reduced more by PoPE treatment. This indicates a complex modulation of the inflammatory response by each extract. For instance,

the reduction of IL-6 would suggests a PePE and GrPE treatment-derived effect against nuclear transcription factor kappa B, which promotes the expression of this interleukin (Ferrari et al., 2016; Lewis et al., 2023). In contrast, the PoPE treatment could have reduced IL-8 production by targeting mitogen-activated protein kinases pathway (Romier-Crouzet et al., 2009), however, this must be confirmed by further research.

The polyphenolic-rich extracts reduced the LPS-induced oxidative stress in Caco-2 and HaCaT cells, showing a protective effect against the changes in the cellular oxidative status. In this sense, the punicalagins from PoPE could have targeted the hydroxyl radicals, the superoxide anions, the hydrogen peroxide, and the nitric oxide (Tanaka et al., 2018; Xu et al., 2015; Yan et al., 2016); supporting the high antioxidant activity of PoPE treatment determined by the DPPH and ABTS assays. Moreover, since the extracts did not show inhibitory effects in cells by the MTT assay at tested doses, it is possible that they acted against ROS without affecting the cell survival. The GrPE and PePE treatments also reduced ROS in Caco-2 and HaCaT cells. Interestingly, the PePE treatment showed the lowest antioxidant activity among the samples when assayed by DPPH and ABTS methods. Anthocyanins and gallic acid, the main compounds in the GrPE and PePE, have previously been associated to stimulate the adaptative cellular response of cells against oxidative stress (Ferrari et al., 2016; Nallathambi et al., 2020; Tanaka et al., 2018), as well as for the upregulation of antioxidant enzymes. Probably, the polyphenolic-rich extracts stimulated the defence mechanisms in Caco-2 and HaCaT cells to counterattack the stressor effect of the LPS.

To determine if the polyphenolic-rich extracts influenced the cellular adaptative response against external stimuli, their effect in the transcription factor Nrf2 in Caco-2 cells was explored. Under normal conditions, Nrf2 remains inactive when complexed with Keap 1 (Kelch-like ECH- associated protein 1); it is activated in response to oxidative stress, being released from Keap1, protecting the cells against stress-induced cell death (Ordonez et al., 2022; Reddy et al., 2009; Tanaka et al., 2018; Yan et al., 2016). The polyphenolic extracts managed to protect the Caco-2 cells from *S. enterica* invasion; however, in Nrf2 KD Caco-2 cells, the percentage of invasive *S. enterica* was higher despite the cells being treated with the same doses of GrPE and PePE. In contrast, the PoPE treatment preserved its effect in Nrf2 KD cells. The same effect was observed in LPS-induced inflammation and oxidative stress, where the GrPE and PePE treatments lost their effectiveness in Nrf2 KD Caco-2 cells, but the PoPE treatment retained its beneficial effect. This suggests that the protective effects of GrPE and PePE are mediated by Nrf2, whereas PoPE operates through Nrf2-independent pathways at tested doses (Fig. 8).

Delphinidin 3-*O*-glucoside in GrPE and gallic acid in PePE, induced cellular adaptative response through Nrf2 activation, leading to the consequent expression of cytoprotective enzymes, according to our *in-silico* results. Thus, similar to other studies, GrPE and PePE treatments induced a cellular adaptative response to pathogenic bacteria and LPS through Nrf2 activation (Ferrari et al., 2016; Gasparrini et al., 2018; Reddy et al., 2009; Xu et al., 2015), inhibiting pro-inflammatory mediators and by decreasing oxidative stress. The punicalagins in PoPE also influence Nrf2 (Yan et al., 2016), which was corroborated by our *in silico* results. The complex phenolic mixture found in the PoPE eliminated free radicals and reduced ROS, as well as the pro-inflammatory cytokines in Nrf2 KD cells (Yan et al., 2016), which is supported by its effect against DPPH and $ABTS^{\bullet+}$ radicals and its TPC and TFC. Additionally, the punicalagins can form complexes with metal ions and proteins, reducing the intracellular ROS generation (Romier-Crouzet et al., 2009; Yan et al., 2016). Therefore, while the PoPE treatment could have activated Nrf2, its effect at stimulating cellular defences against the bacteria and LPS might involve other pathways.

This study highlights the beneficial potential of GrPE, PePE, and PoPE in bacterial-cell host interactions through their pronounced cytoprotective properties in colonic and skin cells, supporting the

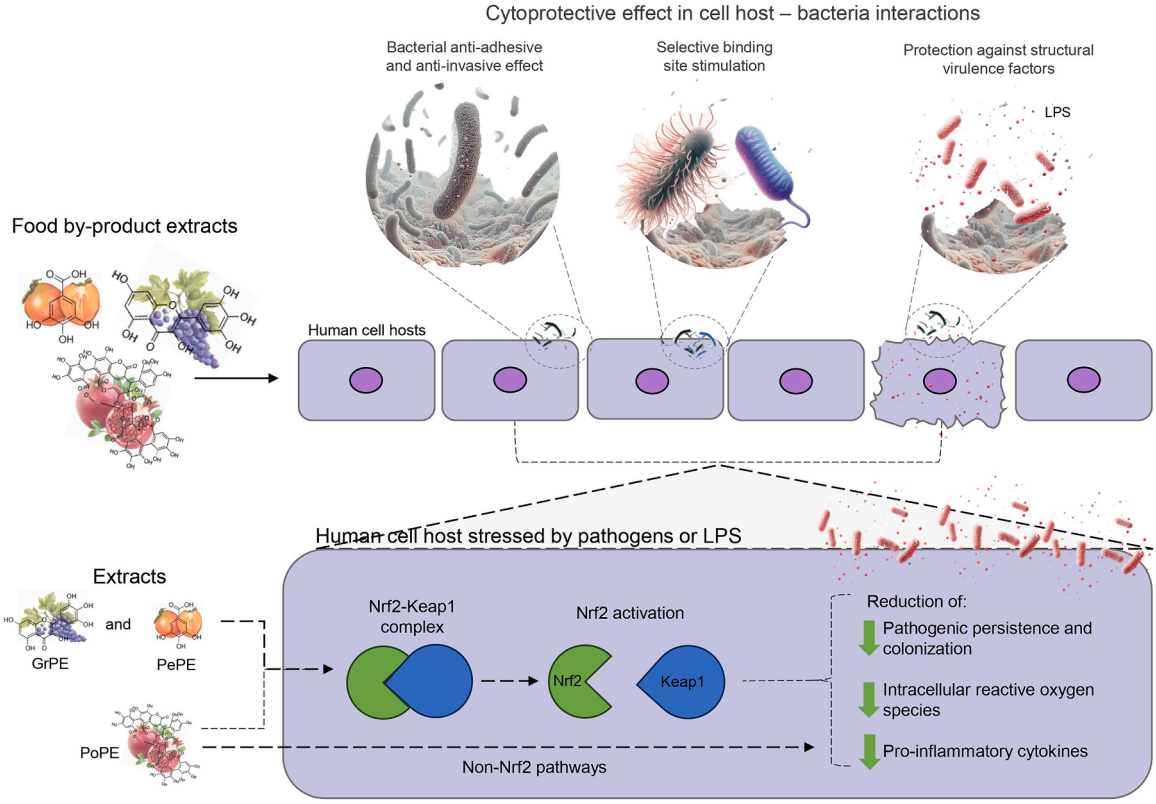

Fig. 8. Mechanism of polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE) to exert cytoprotective effects in cell host during pathogenic bacteria and lipopolysaccharides (LPS) exposition. The effect of GrPE and PePE is dependent of Nrf2 activation while the effect of PoPE is independent of this pathway.

revalorization of fruit by-products as sources of relevant bioactive compounds with antibacterial potential. GrPE and PePE, through delphinidin 3-*O*-glucoside and gallic acid, respectively, demonstrated their beneficial effects by targeting bacterial adhesion, invasion and triggering adaptive cellular defence mechanisms via Nrf2 stimulation, while the effect of PoPE was independent of Nrf2. These promising findings not only endorse the use of these extracts for novel antimicrobial strategies and therapies, including the prevention of bacterial infections, but also underscore their utility in selectively promoting non-pathogenic and commensal strains from the human microbiome. The diverse effects observed at different stages of bacterial infection provide valuable foundation for further in-depth studies using *in vivo* models, unravelling and testing the intricate mechanisms underlying the preventive actions of these extracts against bacterial infections. Furthermore, the TPC found in SICs of extracts showcases their potential applicability of as nutraceuticals for oral or topical administration; however, the therapeutic doses obtained in the study must be confirmed in *in vivo* models to ensure their applicability and effectiveness in the intestinal and skin epithelium for daily use.

Data statement

All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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CRediT authorship contribution statement

Bryan Moreno-Chamba: Writing – original draft, Software, Methodology, Investigation, Data curation. **Julio Salazar-Bermeo:** Investigation, Methodology, Formal analysis, Writing – original draft. **Marta Narváez-Asensio:** Methodology, Investigation, Formal analysis. **Pablo Navarro-Simarro:** Methodology, Investigation. **Domingo Saura:** Validation, Project administration, Conceptualization. **Madalina Neacsu:** Visualization, Supervision, Conceptualization. **Nuria Martí:** Validation, Supervision, Conceptualization. **Manuel Valero:** Writing – review & editing, Project administration, Formal analysis, Data curation, Conceptualization. **María Concepción Martínez-Madrid:** Visualization, Supervision, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.156020.

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