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Effects of grape juice intake on the cell migration properties in overweight women: Modulation mechanisms of cell migration *in vitro* by delphinidin-3-O-glucoside

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ABSTRACT

Overweight and obesity are typical conditions of chronic low-intensity systemic inflammatory responses, and both have become more common in recent decades, which emphasizes the necessity for healthier diet intake. Fruits such as grapes are rich in anthocyanins, one of which is delphinidin, a promising chemopreventive agent with anti-inflammatory properties. Considering that polymorphonuclear cells (PMNs) are rapidly mobilized to tissues when the inflammatory process is initiated, this study aimed to understand the impact of grape juice intake and delphinidin on the migration properties of PMNs. Overweight women ingested 500 mL of grape juice for 28 days, and then lipid and inflammatory profiles, as well as the white blood cell count (WBC), were evaluated. Additionally, the gene expression of inflammatory markers and quantified migration molecules such as CD11/CD18, ICAM-1 and VCAM-1 were evaluated in PMNs. The influence of delphinidin-3-O-glucoside *in vitro* on some migration properties was also evaluated. Grape juice intake did not influence the lipid profile or affect the WBC. However, NFκB gene expression was reduced in PMNs, also reducing the circulating values of IL-8, sICAM-1, and sVCAM-1. The *in vitro* results demonstrated that delphinidin significantly reduced the migration potential of cells and reduced CD11-/CD18-positive cells, the gene expression of ICAM-1, and the phosphorylation and gene expression of NFκB. Additionally, delphinidin also reduced the production of IL-6, IL-8, and CCL2. Grape juice, after 28 days of intervention, influenced some properties related to cell migration, and delphinidin *in vitro* can modify the cell migration properties.

1. Introduction

Anthocyanins, which belong to the flavonoid group of phenolic compounds, are primarily found in plant-based foods, particularly in berries and fruit-derived beverages (Araujo et al., 2021; Tiwari et al., 2009). Anthocyanins are water-soluble pigments, with over 550 recognized types, and six of them (delphinidin, cyanidin, malvidin, pelargonidin, peonidin, and petunidin) are widely distributed in plants (Tiwari et al., 2009). Beyond their role as natural pigments, anthocyanins have been associated with numerous health benefits, including anti-inflammatory, antioxidant, antitumour, and neuroprotective effects (Joseph et al., 2016; Cremonini et al., 2022; Ma et al., 2021). Consequently, there has been a growing interest in exploring the potential of bioactive compounds in foods that can promote health and well-being.

Fruits such as grapes are rich in phenolic compounds distributed in different parts of the fruit, with the skin being the main source, followed by the seeds and pulp (Tan et al., 2021; Latruffe and Rifler, 2013). In grape juice, the glucoside forms of anthocyanins are responsible for the

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red colour (Sabra et al., 2021; Cao et al., 2001). In addition, numerous reports confirm the positive effect of grape juices in the treatment of various disorders, modulating several cellular processes related to the inflammatory response (Joseph et al., 2016; Cremonini et al., 2022; Xu et al., 2012; Bøhn et al., 2021; Martins et al., 2020).

The incidences of overweight and obesity have increased globally in recent decades, mainly due to increased consumption of ultra-processed foods, refined carbohydrates, and saturated fatty acids, which cause diseases associated with chronic low-grade inflammation (Heredia et al., 2012; Rodríguez-Hernández et al., 2013; Cena and Calder, 2020). Thus, the consumption of healthier diets, giving attention to the intake of specific food groups, or overarching dietary patterns, can influence health benefits, promoting the prevention of low-grade inflammatory diseases (Heredia et al., 2012; Cena and Calder, 2020).

Additionally, when the inflammatory process is discussed, it must be clear that it is a complex process, and each step of the inflammatory response is mediated by several molecules that activate different signalling pathways. Of particular importance is the pathway that triggers the activation of the transcription factor NFκB, which plays pivotal roles in the expression of several pro-inflammatory genes, including genes encoding cytokines and chemokines that regulate the migration of WBCs from circulation to inflamed sites (Luster et al., 2005; Mitchell et al., 2016). In this way, leukocyte migration is essential for effective host responses to tissue damage and is initially orchestrated by a dynamic milieu of adhesion molecules, such as selectins and integrins, and chemotactic factors, such as CXC chemokines, which are a large family of potent chemoattractants (Ma et al., 2021. Luster et al., 2005; Mitchell et al., 2016).

Therefore, this study aimed to investigate whether daily consumption of whole grape juice for 28 days could modulate bloodstream cell migration in overweight women. Additionally, knowing that the main anthocyanin present in the grape juice used was delphinidin, the study also investigated *in vitro* some of the mechanisms behind cell migration control.

2. Materials & methods

2.1. Grape juice characterization

The experimental product used for the supplementation was a commercial grape juice characterized as a natural, integral and nonalcoholic produced from a mix of Bordô, Concord, Isabel and Violet grapes from southern Brazil. All the studies were performed with a single batch of whole grape juice, without the addition of water, sugar and preservatives. According to the producer, 100 mL of grape juice contains 73 kcal and 17 g of carbohydrates. The soluble sugar composition was analysed using a CarboPac column (250 mm × 4 mm) with its corresponding guard column (Dionex, Sunnyvale, CA, USA) in a DX 500 high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) system (Dionex, DX500, Sunnyvale, CA, USA). The mobile phase consisted of 18 mM NaOH at a flow rate of 1 mL min^{-1} for 25 min. The quantification was based on an external calibration using sucrose, glucose, and fructose (Cordenunsi et al., 2008).

The total phenolic content was quantified according to the Folin − Ciocalteu method (Swain and Hillis, 1959), and the absorbance was measured at 763 nm. The results were expressed as mg of gallic acid equivalent per mL (mg GAE/mL). Anthocyanin was analysed by HPLC-DAD and LC-ESI-MS/MS (Sestari et al., 2014) after solid phase extraction. Briefly, grape juice was passed through a polyamide column (1 g) (CC 6, Macherey-Nagel Gmbh and Co., Duren, Germany) and eluted with 0.3 % HCl in methanol. The eluates were completely dried using a rotary evaporator under vacuum at 40 °C, resuspended in methanol, and filtered through a 0.45 μm PTFE filter (Millipore Ltd.). LC-ESI-MS/MS was performed using a Prominence liquid chromatograph (Shimadzu, Japan) linked to an ion trap Esquires-LC mass spectrometer (Bruker Daltonics, Billerica, MA, USA) with an electrospray ionization (ESI) interface. The column used was a 5 μm Prodigy ODS3 column (4.60 × 250 mm) (Phenomenex Ltd., UK). The mobile phase consisted of solvent A, 0.5 % formic acid in water, and solvent B, 0.5 % formic acid in acetonitrile. The solvent gradient was 10 % B at the beginning, 10 % at 5 min, 20 % at 15 min, 25 % at 25 min, 35 % at 33 min, 50 % at 38 min, 90 % at 43–44 min, and 10 % at 45 min, with a flow rate of 1 mL/min at 25 °C. The flow rate was changed to 0.2 mL/min for application to the mass spectrometer. The mass spectrometer operating conditions were a collision energy of 3500 V and a capillary temperature of 275 °C. The analysis was carried out using a full scan from m/z 100 to 1500 in positive mode. The supplemental data show the chromatogram of grape juice obtained by LC-ESI-MS/MS (**Supplementary Figure S1**).

2.2. Subjects and study design

This is an interventional study where twenty overweight women with a body mass index (BMI) between 25 and 29.9 kg/m^2 and an age between 18 and 40 years old were recruited. The studýs recruitment occurred at the Health Center "Geraldo Horacio de Paula Souza", School of Public Health, University of São Paulo, Brazil. The exclusion criteria were as follows: male; aged less than 18 or more than 40 years old; BMI less than 25 or more than 29.9 kg/m^2; gastrointestinal disturbances or diseases; athlete; metabolic diseases; altered plasma lipid profile; altered systolic or diastolic blood pressure; use of anti-inflammatory drugs; use of supplements; use of medicines that could affect the intestinal microbiota; regular consumption of grape juice, red wine or red fruits; smokers; pregnancy. Additionally, volunteers who, during the intervention, started to participate in another study, consumed alcohol in this period, or presented haemoglobin concentrations lower than 12 g/dL were excluded from this current study. Volunteers could also not practice physical exercise 48 h before blood collection, and on the day of blood collection, 12 h of fasting were stipulated.

Volunteers received 500 mL of grape juice for daily consumption for 28 days, and data and samples were collected at 0, 14, and 28 days after intervention. Body weight, BMI, and waist and hip circumference were measured. Blood samples (30 mL) were collected in tubes containing EDTA for cellular analyses or in clot-activator tubes with gel for biochemical analyses.

This study received ethical approval from the Committee for Ethics in Research of the School of Public Health, University of Sao Paulo, Brazil (Approval: 69382117.6.0000.5421) and registered on the Brazilian Registry of Clinical Trials (U1111-1241-0556). All volunteers provided written informed consent before enrolment in the study.

2.3. White blood cell count, biochemical and cytokine analysis

Tubes collected with EDTA were used for white blood cell count (WBC) analyses and flow cytometry analyses. Additionally, polymorphonuclear cells (PMNs) were isolated and used for mRNA quantification. Serum samples collected in tubes without anticoagulant were separated for biochemical and cytokine quantification.

WBC was performed by automated equipment (Horiba ABX, France), and leukocyte differential was checked on blood smears (Wright-Giemsa stain; Merck, Germany). For cytometry analysis of bloodstream populations, samples collected in EDTA tubes were centrifuged at 300*g* for 10 min at room temperature, and plasma was discharged.

For flow cytometry analyses, red blood cells were first removed using Cell Lysis Buffer (BD Pharmingen, USA) according to the manufacturer's instructions. Then, using flow cytometry, it was determined whether the white blood cells had been positively labelled with anti-CD4 (PE) (#555347; BD Biosciences, USA), anti-CD8 (APC) (#553035; BD Biosciences, USA), anti-CD14 (APC) (#555399; BD Biosciences, USA), anti-CD11b (PE-Cy7) (#552850; BD Biosciences, USA) and anti-CD18 (FITC) (#555923; BD Biosciences, USA). As a negative control, the fluorescence minus one (FMO) strategy was used. Antibodies were incubated at 4 °C (dark) for 1 h, cells were washed with PBS, and the pellet was then

resuspended in 0.5 mL of PBS. The supplemental data show the cytometry strategy (**Supplementary Figure S2**). A total of 10,000 cells were processed on a FACS Canto II (FACScan; Becton Dickinson, USA), and FlowJo software (Tree Star Inc., USA) was used to analyse the cell populations.

Tubes collected without anticoagulant were centrifuged at 300*g* for 10 min at room temperature, and serum was separated and frozen at −80 °C until analysis of glucose, lipid profile, aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), C-reactive protein (CRP), creatinine and blood urea nitrogen (BUN). The Clinical Analysis Laboratory of University Hospital of São Paulo, Brazil processed the samples by an automated analyser (Roche, Cobas® 6000, Switzerland).

Additionally, serum was also used for interleukin (IL)-6, IL-8, IL-10, soluble vascular cell adhesion molecule-1 (sVCAM-1), and soluble intercellular adhesion molecule-1 (sICAM-1), and quantification was assessed using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA) according to the manufacturer's instructions.

2.4. Analysis of gene expression from polymorphonuclear cells (PMNs) isolated from the bloodstream

Blood cell samples from the volunteers were collected in EDTA tubes as previously described, and the PMNs were isolated from these samples following the Percoll method (Kuhns et al., 2015). The Percoll method obtained a PMN population composed of 95.79 % ± 1.6 neutrophils, 2.92 % ± 0.91 eosinophils, 0.14 % ± 0.36 basophils and 1.14 % ± 0.94 peripheral blood mononuclear cells (PBMCs), and more than 95 % of these cells were viable, as evaluated by the trypan blue exclusion test, after the Percoll procedure. After obtaining PMNs, total RNA was extracted using RNeasy (Qiagen, USA) and reverse transcribed into cDNA using a High-Capacity cDNA kit (Applied Biosystems, USA). A total of 50 ng of RNA was used, and the cDNA samples were amplified in TaqMan® Fast Advanced master mix (Applied Biosystems, USA) with the primer set (Applied Biosystems, USA) for TNF-α (Hs00174128_m1), *IL-1β* (Hs01555410_m1), *IL-8* (*CXCL8*; Hs00174103_m1), *NFκB* (Hs00765730_m1), *NFκBIA* (*IκBα* Hs00153283_m1) and 18S gene expression as an internal control (RNA18S5; Hs03928990_g1). StepOnePlus™ (Applied Biosystems, USA) real-time PCR was used to evaluate gene expression according to the ΔΔCt method (Livak and Schmittgen, 2001).

2.5. Cell culture of C1498 cells and treatments to choose delphinidin dosages

C1498 cells were obtained from the American Type Culture Collection (ATCC® TIB-49TM). C1498 cells are a murine myeloid leukaemia cell line isolated from a C57BL/6J mouse composed of monoblasts and myeloblasts (Mopin et al., 2016). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10 % glucose (Vitrocell®, Brazil) and 10 % foetal bovine serum (FBS) (Vitrocell®, Brazil) as well as HEPES buffer (2 g/L), 1 % streptomycin (Sigma-Aldrich®, USA) and penicillin (Sigma-Aldrich®, USA) and a pH range of 7.2 to 7.4. These cells were kept in an environment of 37 °C with 5 % CO_2.

C1498 cells were plated and cultivated for 24 h with or without delphinidin (delphinidin-3-O-β-glucoside; cat. 73705, Sigma-Aldrich®, USA) at doses of 0, 3, 6, 12, 25, 50, 100 and 200 μM (Manach et al., 2004; Goszcz et al., 2017; Wu et al., 2006) to choose the best dosage based on the MTT method, apoptosis taxes and cell cycle analyses. Additionally, to mimic an inflammatory environment, cells were stimulated with LPS (1.25 μg/mL of lipopolysaccharide; 055: B5, Sigma-Aldrich®, USA) (Fock et al., 2007; Zhong et al., 2021; Neves et al., 2023).

2.6. MTT assay

Cell cytotoxicity was determined by the MTT-tetrazolium (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide); Sigma-Aldrich®, USA) assay (Mosmann, 1983). C1498 cells (1×10^4) were plated in 96-well plates and cultivated for 24 h with or without delphinidin and stimulated or not with LPS. After 24 h, 50 μL of an MTT colorimetric solution (0.5 mg/mL) was added and incubated for more than 4 h. After this time, 500 μL of 10 % sodium dodecyl sulfate (SDS) was added and incubated in the dark for 2 h. The absorbance of each well was determined at 570 nm, and the results were analysed.

2.7. Cellular apoptosis taxes and cell cycle evaluation

For cellular apoptosis evaluation, C1498 cells (1×10^6 cells /mL) were cultivated for 24 h with or without delphinidin and stimulated or not with LPS. After the experimental periods and stimulus, cells were collected and rinsed with PBS. After centrifugation at 300*g* for 10 min at 4 °C, the pellet was suspended in 50 μL of annexin buffer, and 3 μL of annexin-V (Becton & Dickinson, USA) plus 5 μL of 7-amino-actinomycin D (7-AAD) (Becton & Dickinson, USA) were added. The cells were incubated for 20 min (protected from light). After that time, the cells were centrifuged at 300*g* for 5 min, and the pellet was resuspended in 200 μL of annexin buffer for data acquisition. For cell cycle evaluation, cells cultivated as previously described were collected and fixed with 70 % ethanol, followed by centrifugation (300*g* for 5 min). The pellet was resuspended in 4 mg/mL ribonuclease A (#R2011, US Biological, USA) plus 4 μL propidium iodide (PI) for 45 min (protected from light). The cell cycle was evaluated by quantifying the histograms of the G1/G0 and S/G2/M phases (**Supplementary Figure S3**). A total of 20,000 cells were acquired on a FACS Canto II (FACScan, Becton Dickinson, USA), and FlowJo software (Tree Star Inc., USA) was used for data analysis.

2.8. Cytokine production

C1498 cells (1×10^6 cells/mL) were cultivated for 24 h with or without 50 μM delphinidin and stimulated or not with LPS. After this time, supernatants were collected, and the production of IL-1β, IL-6, IL-8 and CCL-2 was assessed by ELISA (R&D Systems, USA) according to the manufacturer's instructions.

2.9. In vitro migration assay

To assess how delphinidin may affect the migration of haematopoietic cells, 1×10^6 C1498 cells were seeded in 24-well plates containing transwells with pores of 8 μm (product number 353097; Corning, USA). These C1498 cells were seeded in the upper chamber with 300 μL of DMEM without any supplementation of FBS in the presence or absence of 50 μM delphinidin, while 300 μL of media was placed in the lower chambers. Additionally, to test whether delphinidin may affect the migration of C1498 cells, they were also challenged. In the first case, 1.25 μg/mL LPS was also added to the upper chamber, and in the second case, the chemotactic agent N-formylmethionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich®, USA) was added at a concentration of 0.1 μM to the lower chambers. After plating, the cells were incubated for 6 h and, past the incubation period, aspirated and stained with trypan blue for manual counting using a Neubauer chamber.

2.10. Flow cytometry

To access integrins CD11 and CD18 on the surface of C1498 cells flow cytometry was used. First, C1498 cells (1×10^6 cells/mL) were cultivated for 24 h with or without 50 μM delphinidin and stimulated or not with LPS. A total of 1×10^6 cells/mL were incubated with anti-CD11b (FITC) (#553310; BD Biosciences, USA) and anti-CD18 (PE) (#553293; BD Biosciences, USA) for 30 min at 4 °C in the dark, and after

this period, the cells were washed with PBS and resuspended in 0.5 mL of PBS. A total of 10,000 cells were acquired on a FACS Canto II (FACScan; Becton Dickinson, USA) and analysed using FlowJo software (Tree Star Inc., USA). The flow cytometry strategy used in this *in vitro* step is shown in the supplementary information (**Supplementary Figure S4**).

2.11. Western blot analysis

For the analyses of the expression of the transcription factor NFκB in C1498 cells, Western blotting was performed. A total of 1×10^6 C1498 cells per mL were cultivated in the presence or absence of 50 μM delphinidin for 24 h. After this period, C1498 cells were stimulated or not with 1.25 μg/mL LPS for 30 min. After that, the cells were washed with PBS three times and then lysed with RIPA lysis and extraction buffer (Thermo Fisher Scientific Inc., USA) containing 2 μg/mL aprotinin, 1 μg/mL leupeptin, 100 μg/mL PMSF and 0.5 mM EDTA. The supernatant was collected after centrifuging at 20,000*g* for 15 min at 4 °C with 5 × Laemmli buffer (1 M Tris HCl, 10 % 2-mercaptoethanol, 10 % SDS, 50 % glycerol and 0.01 % bromophenol blue) and boiled at + 95–100 °C for 5 min. The BCA Protein Assay Kit (Pierce Biotechnology, Inc., USA) was used to quantify the protein amount, and 20 μg of protein was loaded on 10 % SDS-polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corporation, USA). The membranes were incubated overnight with primary antibodies (1:1,000) against NFκB (sc-109, Santa Cruz Biotechnology, USA) and phosphorylated NFκB (sc-33039, Santa Cruz Biotechnology, USA). After this time, the membrane was washed with PBS and incubated with a secondary antibody for 1 h (1:1,000; HRP, #7074, Cell Signaling Technology, USA). Membranes were washed three times with TBST (Tris-buffered saline, 0.1 % Tween 20), and bands were revealed using the ECL kit (GE Healthcare, USA). The membranes were assessed using a detection system (ImageQuant™ 400, GE Healthcare, USA), and the results were expressed in relation to β-actin (1:50,000; A3854 Sigma-Aldrich®, USA).

2.12. RNA isolation and quantitative real-time PCR of C1498 cells

C1498 cells (1×10^6 cells /mL) were cultivated for 24 h with or without 50 μM delphinidin and stimulated or not with LPS. After this period, cells were collected, and total RNA extraction and reverse transcription into cDNA were performed as described previously. The primer sets (Applied Biosystems, USA) used were iNOS (*nos2*; Mm00440502_m1), STAT-3 (*stat3*; Mm01219775_m1), AKT-1 (*akt1*; Mm01331626_m1), NFκB (*nfkb2*; Mm00479807_m1), ICAM-1 (*icam1*; Mm00516023_m1) and PECAM-1 (*pecam1*; Mm01242584_m1), and 18S gene expression was used as an internal control (*rn18s*; Mm03928990_g1). StepOnePlus™ (Applied Biosystems, USA) real-time PCR was used to evaluate gene expression according to the ΔΔCt method (Livak and Schmittgen, 2001).

2.13. Statistical analysis

A homoscedasticity test was used to evaluate whether the data were normally distributed, and according to the results, variables are summarized as the mean ± standard deviation (normal distribution). The *in vivo* dataset was first evaluated in relation to the *baseline* (time 1) using one-way ANOVA with Dunnetts post hoc test. The *in vitro* results were evaluated by one-way or two-way ANOVA with Tukeýs post hoc test. All results were considered statistically significant when $p < 0.05$, for a significance level of 95 %. Statistical analyses were performed using GraphPad Prism® software (GraphPad Software Inc., USA).

3. Results

3.1. Interventional study

The concentration of total phenolics in the grape juice used in the study was 235.6 mg of equivalent gallic acid (mg AGE/100 mL) of juice. In this way, the volunteers consumed approximately 1178.0 mg AGE per day by drinking 500 mL of grape juice. Additionally, among the anthocyanins found in the grape juice used, delphinidin-3-glucoside was the most abundant (84.80 mg/L) (Table 1).

The final number of volunteers evaluated was 14. Initially, 20 volunteers were recruited, but 6 of them were excluded from the study due to one pregnancy, one used anti-inflammatory medicines, one did not show up in the third period of the study, and three did not follow the recommended protocol.

The grape juice intervention did not result in changes in values related to body composition and biochemical parameters (Table 2). Additionally, serum C-reactive protein (CRP), IL-6, and IL-10 concentrations did not show differences among the time points studied, but the IL-8 concentrations showed reduced values after 14 and 28 days of grape juice intake. Although sICAM-1 and sVCAM-1 did not show differences after 14 days, they showed reduced values after 28 days of grape juice intake in comparison to the baseline values (Table 2).

The grape juice intervention did not result in changes in the WBC count or in the blood cell expression of CD11, CD18, or CD14. Additionally, the CD4/CD8 ratio did not change after grape juice intervention (Table 3). Regarding gene expression, PMNs were isolated by the Percoll method, consisting of a population of 95.79 % ± 1.6 neutrophils, and the results in this population did not show differences in the expression of *IL-1β*, *IL-8*, *TNFa*, *NFκB*, and *NFκBIA*. However, the expression of *NFκB* was lower after 28 days in comparison to the baseline (Table 3).

3.2. Cell viability and cell cycle of C1498 cells cultivated with delphinidin

Among the dosages analysed, the highest dose tested that did not increase the cytotoxicity was 50 μM (Fig. 1A). Additionally, doses of 200 μM delphinidin increased apoptosis and decreased viability in cells cultivated with or without lipopolysaccharides (LPS) (Fig. 1B and 1C), and doses of 100 μM delphinidin in cells cultivated with LPS increased apoptosis (Fig. 1C), demonstrating that a dose of 50 μM delphinidin had the least impact on cell viability.

Regarding the cell cycle, it was also possible to see that 200 μM delphinidin affects the percentage of cells stimulated or not stimulated with LPS in the G0/G1 and S/G2/M phases of the cell cycle in comparison to cells cultured without delphinidin (Fig. 1D and 1E), and cells cultivated with 100 μM delphinidin and not stimulated with LPS showed an increased percentage of cells in the G0/G1 cell cycle phase in comparison to cells cultivated without delphinidin (Fig. 1D).

Table 1
Nutritional composition of grape juice.

Nutritional composition	(500 mL)
Energy (kcal)	365.00
Carbohydrate (g)	85.00
Total sugars (g)	68.90
Glucose (g)	31.80
Fructose (g)	37.10
Sucrose (g)	0.00
Total phenolics (mg GAE)	1178.00
Delphinidin 3-glucoside (mg)	42.40
Malvidin 3-glucoside (mg)	11.60
Petunidin 3-glucoside (mg)	9.55
Cyanidin 3-glucoside (mg)	7.30
Peonidin 3-glucoside (mg)	5.10

Table 2
Body composition, biochemical parameters and cytokines profile of the patients at baseline, 14 and 28 days after the intake of grape juice.

	Baseline	14 Days	28 Days
Weight (kg)	69.9 ± 6.6	70.4 ± 7.0	70.4 ± 6.1
Body mass index (kg/m^2)	27.3 ± 1.2	27.2 ± 1.3	27.2 ± 1.1
Waist circumference (cm)	88.7 ± 5.7	88.5 ± 3.9	88.9 ± 4.44
Waist-to-hip ratio	0.84 ± 0.04	0.83 ± 0.04	0.83 ± 0.05
Glucose (mg/dL)	78.3 ± 10.5	78.4 ± 5.5	81.7 ± 10.6
Total cholesterol (mg/dL)	161.1 ± 45.3	149.1 ± 47.5	161.3 ± 36.6
HDL-cholesterol (mg/dL)	42.2 ± 11.2	43.8 ± 12.9	49.1 ± 11.7
ALT (U/L)	10.8 ± 4.8	12.3 ± 6.0	13.1 ± 3.5
AST (U/L)	14.5 ± 2.9	17.5 ± 9.6	17.3 ± 5.4
BUN (mg/dL)	19.0 ± 6.1	19.8 ± 4.9	20.1 ± 5.3
Creatinine (mg/dL)	0.51 ± 0.21	0.54 ± 0.19	0.61 ± 0.19
Sodium (mEq/L)	139.0 ± 4.8	143.4 ± 3.9	140.1 ± 2.4
Potassium (mEq/L)	4.03 ± 0.26	3.87 ± 0.66	4.15 ± 0.42
CRP (mg/L)	2.66 ± 1.99	2.35 ± 2.04	2.21 ± 2.57
IL-6 (pg/mL)	16.6 ± 5.7	15.4 ± 4.6	16.8 ± 5.8
IL-8 (pg/mL)	5.7 ± 1.1	4.7 ± 0.9**	4.9 ± 0.8**
IL-10 (pg/mL)	21.5 ± 3.0	20.8 ± 2.6	20.4 ± 3.1
sICAM (ng/mL)	240.7 ± 50.3	229.4 ± 40.3	212.7 ± 50.9***
sVCAM (ng/mL)	632.4 ± 101.8	570.5 ± 99.4	498.6 ± 108.9*

Results are expressed as mean ± standard deviation. Asterisks indicate significant differences between 14 or 28 days in comparison to baseline. *($p \leq 0.05$), ** ($p \leq 0.01$) and ***($p \leq 0.001$). $n = 14$. n represents the number of volunteers participating in the study.

Table 3
White blood cell count and differential, peripherical CD11 and CD18 quantification and mRNA expression on PMN of the patients at baseline, 14 and 28 days after the intake of grape juice.

	Baseline	14 Days	28 Days
White blood Cell (x10^3/mm^3)	7.62 ± 1.74	7.65 ± 1.77	7.64 ± 2.36
Band cells (mm^3)	143 ± 99	130 ± 107	113 ± 79
Neutrophils (mm^3)	4839 ± 1424	4570 ± 1355	4561 ± 1329
Eosinophils (mm^3)	109 ± 67	113 ± 79	139 ± 143
Basophils (mm^3)	19 ± 39	29 ± 36	33 ± 43
Lymphocytes (mm^3)	2111 ± 453	2355 ± 529	2374 ± 835
Monocytes (mm^3)	398 ± 116	458 ± 113	421 ± 131
CD11 (MFI)	7261 ± 1945	6388 ± 1661	6384 ± 2036
CD18 (MFI)	1686 ± 857	1685 ± 728	1464 ± 597
CD14 (MFI)	834 ± 320	844 ± 165	804 ± 231
CD4/CD8 ratio	1.95 ± 0.77	2.26 ± 1.39	2.11 ± 0.99
mRNA *IL-1β* (relative to 18S)	1.22 ± 0.46	1.09 ± 0.43	1.01 ± 0.45
mRNA *IL-8* (relative to 18S)	1.48 ± 1.26	1.11 ± 0.84	1.27 ± 1.13
mRNA *TNFα* (relative to 18S)	1.26 ± 0.62	1.08 ± 0.43	1.08 ± 0.39
mRNA *NFκB* (relative to 18S)	1.05 ± 0.39	0.94 ± 0.39	0.82 ± 0.27*
mRNA *NFκBIA* (relative to 18S)	1.43 ± 1.10	1.41 ± 0.98	1.39 ± 0.99

Results are expressed as mean ± standard deviation. Asterisks indicate significant differences between 28 days in comparison to baseline. *($p \leq 0.05$). $n = 14$. n represents the number of volunteers participating in the study.

3.3. Soluble factor production by C1498 cells after LPS stimulation in the presence of delphinidin

After the dosage of 50 μM delphinidin was selected, C1498 cells were cultivated with delphinidin, and the production of soluble factors, such as IL-6, IL-8, CCL-2, and IL-1β, was measured when the cells were either stimulated with LPS or not (Fig. 2A–D). The production of IL-6 (Fig. 2A) showed that cells cultivated with LPS increased their production, and delphinidin was able to attenuate the production of this cytokine. Similar results were observed for the production of IL-8 and CCL-2, where increased production of these cytokines was observed after LPS stimulation, showing that delphinidin can reduce this production (Fig. 2B and 2C). Regarding IL-1β, although the cells stimulated with LPS increase their production, it was not observed that delphinidin in these cells can attenuate the production of IL-1β (Fig. 2D).

Considering that NFκB is a transcription factor that plays an important role in the inflammatory response, we opted to measure the phosphorylation of this protein by presenting the p-NFκB/NFκB ratio (Fig. 2E). According to the findings, LPS increased the p-NFκB/NFκB ratio, but this effect did not occur when cells were cultivated with delphinidin (Fig. 2E).

3.4. Effect of delphinidin on C1498 cell migration and CD11 and CD18 expression

As shown in Fig. 3A, cells stimulated with LPS or with N-formylmethionyl-leucyl-phenylalanine (fMLP) migrated more, and delphinidin was able to reduce the migration tax. Additionally, CD11 and CD18 expression on C1498 cells was reduced in cells cultured with delphinidin and stimulated with LPS in comparison to cells stimulated with LPS but cultured without delphinidin (Fig. 3B).

3.5. Effect of delphinidin on C1498 expression of genes related to inflammatory and migration signalling

After determining how delphinidin affects cytokine production and cell migration, we decided to determine how delphinidin might influence C1498 cell function at the mRNA level (Fig. 3C-H). Gene expression of *akt1, nos2* and *stat3* was not affected by delphinidin in the presence or absence of LPS stimulus (Fig. 3C-E). Regarding the gene expression of *pecam1*, although LPS increased this expression in C1498 cells, delphinidin was not able to reduce this expression (Fig. 3F). However, regarding *icam*1 expression, LPS increased its expression, and in cells cultivated with delphinidin and stimulated with LPS, its expression was attenuated (Fig. 3G). Additionally, *nfkb* gene expression was also increased after LPS, and delphinidin was able to reduce this expression even in C1498 cells stimulated with LPS (Fig. 3H).

4. Discussion

This study was developed in two parts, one *in vivo* and the other *in vitro*, intending to understand some points related to cell migration and anti-inflammatory mechanisms associated with grape juice intake and delphinidin supplementation, respectively.

Concerning the *in vivo* step of this study, we did not observe any changes in the anthropometric and biochemical parameters evaluated during the period of the study. Some studies show that after the acute and/or chronic ingestion of grape juice, there are benefits for the cardiovascular system, such as cholesterol reduction and increased circulating high-density lipoprotein (HDL), as well as the control of diabetes, among other aspects associated with metabolic syndrome (Barona et al., 2012; Graf et al., 2013).

Studies conducted in animal models and humans show a positive effect associated with grape juice ingestion and a decrease in biomarkers of inflammation (Ma et al., 2021; Graf et al., 2013; Barona et al., 2012; Bøhn et al., 2021). In this way, the current study observed reduced expression of NFκB in PMNs of volunteers after 28 days of grape juice intervention. However, no significant differences were found in the CRP, IL-6, and IL-10 serum levels, or in the gene expression of IL-1, IL-8, TNF-α and IκBα in PMNs. Additionally, no differences in the WBC count or in the expression of CD11 and CD18 were observed in this study.

On the other hand, when evaluating soluble factors related to mechanisms involved in leukocyte migration, it was observed that grape juice can modulate these factors, such as IL-8, sICAM-1 and sVCAM-1. In this way, the interaction of leukocytes with endothelial adhesion molecules is an important mechanism for leukocyte migration (Pascalis and Etienne-Manneville, 2017; Vestweber, 2015), and upregulation of ICAM-1 and VCAM-1, as well as the chemoattractant IL-8, is observed during inflammatory responses, favouring leukocyte cell migration (Vestweber, 2015; Cambier et al., 2023), where adhesion molecules on leukocytes, such as integrins CD11/CD18, interact with ICAM-1 and very late antigen-4 (VLA-4) interacts with VCAM-1 (Bouti et al., 2021; Yusuf-Makagiansar et al., 2002). Additionally, liberation of circulating

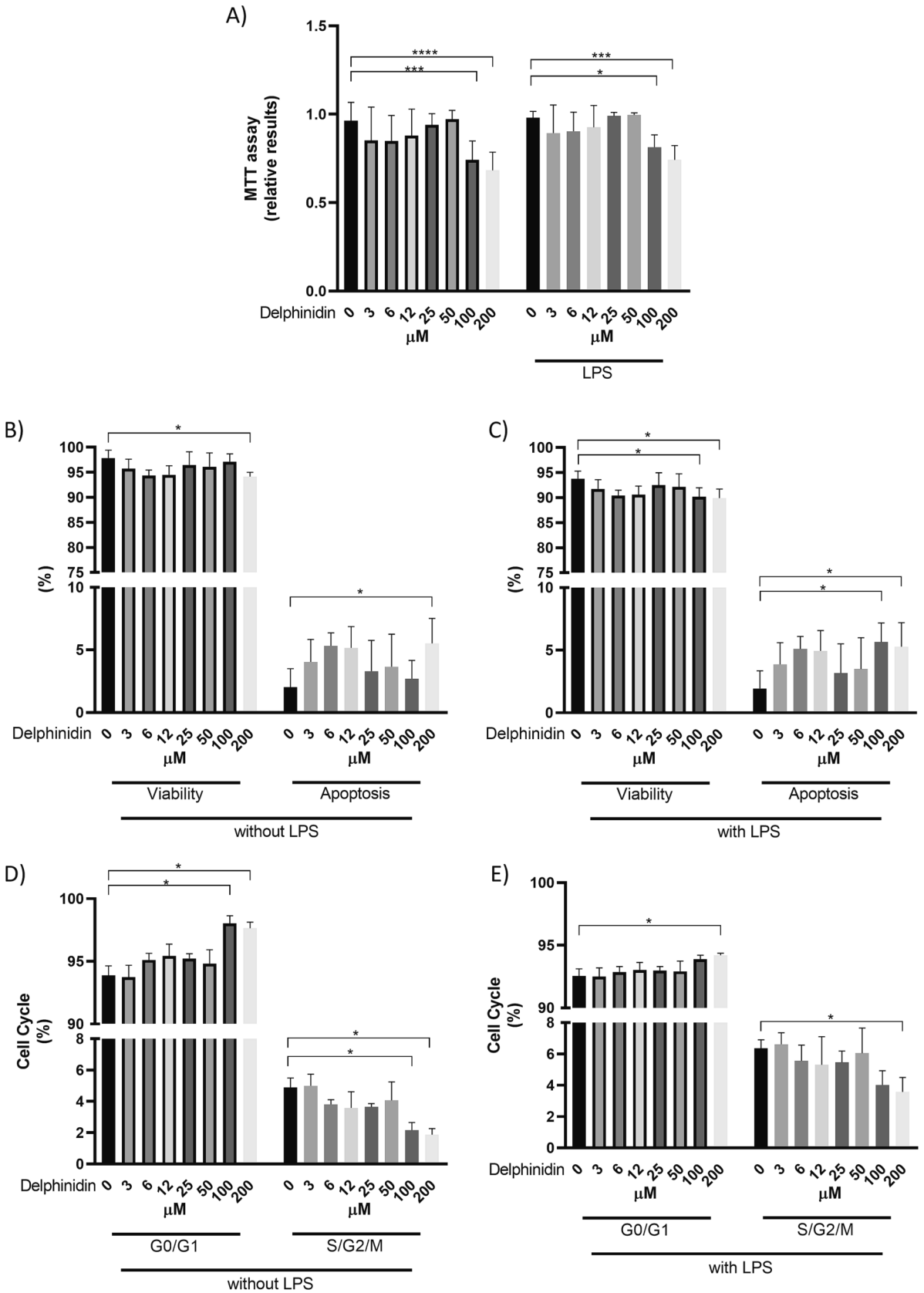

Fig. 1. The C1498 cells were cultivated with or without delphinidin and stimulated or not with LPS. Results of (**A**) cell cytotoxicity evaluated by MTT assay (n = 9), (**B**) Cell viability and apoptosis of cells not stimulated with LPS (n = 6), (**C**) Cell viability and apoptosis of cells stimulated with LPS (n = 6), (**D**) Cell cycle phases of cells not stimulated with LPS (n = 6), (**E**) Cell cycle phases of cells stimulated with LPS (n = 6). Results are expressed as mean ± standard deviation. Asterisks indicate significant differences. *($p \leq 0.05$), ***($p \leq 0.001$) and ****($p \leq 0.0001$). *n* represents the number of samples evaluated.

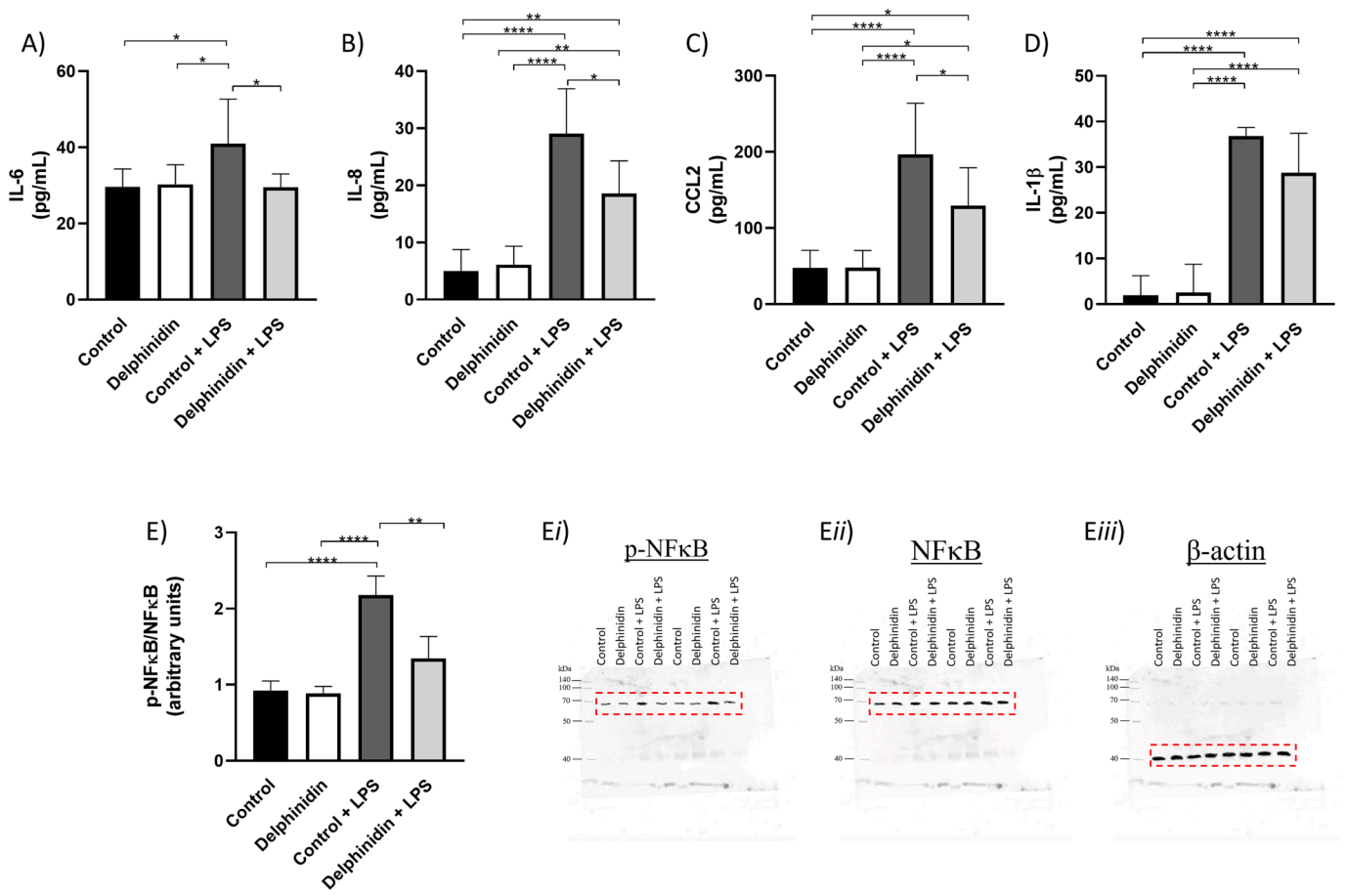

Fig. 2. The C1498 cells were cultivated with or without delphinidin and stimulated or not with LPS. Results of (**A**) IL-6, (**B**) IL-8, (**C**) CCL2 and (**D**) IL-1β production. (**E**) p-NFκB/NFκB ratio protein expression, (***Ei-Eiii***) representative membrane markers. The results are expressed as the mean ± standard deviation (n = 6). Asterisks indicate significant differences. *($p \leq 0.05$), **($p \leq 0.01$) and ****($p \leq 0.0001$). *n* represents the number of samples evaluated.

isoforms of ICAM-1 (sICAM-1) and VCAM-1 (sVCAM-1) are released from the cell surface, and as reported in the literature, increased concentrations of sVCAM-1 and sICAM-1 can be found in obese people (Glowinska et al., 2005).

Moreover, according to the study by Chen et al. (2011), delphinidin significantly attenuated the expression of ICAM-1 in a mechanism related to inhibition of the NFκB pathway. Additionally, the literature has shown a potent antioxidant property of anthocyanin, inhibiting the expression of ICAM-1 and VCAM-1 (Watson and Schönlau, 2015). Delphinidin is an anthocyanin, and although studies have already shown effects on the inflammatory response (Ma et al., 2021; Sabra et al., 2021; Cremonini et al., 2022), little is known about the effects of delphinidin on cell migration, even knowing that cell migration is an important aspect related to the inflammatory response (Cambier et al., 2023; Yusuf-Makagiansar et al., 2002). Consequently, as we observed that delphinidin was the most present anthocyanin in the grape juice used, we set out to elucidate some *in vitro* properties and effects of delphinidin related to migration and the inflammatory response.

Thus, to test our hypothesis, we used a myeloid cell lineage with neutrophilic characteristics and studied several aspects of the ability of delphinidin to modulate these cells when stimulated with LPS. First, the dose to be used was established by several experiments related to cytotoxicity, cell viability, cell cycle and IC_{50} calculation, and the dose of delphinidin chosen was 50 μM, which is also in accordance with the literature showing that higher concentrations are toxic for cells (Manach et al., 2004; Hafeez et al., 2008; Goszcz et al., 2017).

Additionally, delphinidin *in vitro*, attenuates IL-6, IL-8 and CCL2 production, NFκB phosphorylation, CD11b/CD18 expression, cell migration capacity and ICAM-1 gene expression, which demonstrates significant anti-inflammatory potential.

The unregulated influx of PMNs into tissues is a pathological hallmark of several chronic inflammatory diseases (Herrero-Cervera et al., 2022; Vestweber, 2015; Glowinska et al., 2005). The trafficking of PMNs to local inflammation starts with their rolling in the vascular endothelium mediated by adhesion interactions between selectins and their glycosylated ligands (Vestweber, 2015; Herrero-Cervera et al., 2022). The PMN rhythm during rolling is triggered by chemokines/cytokines and mediated by the binding of CD11 and CD18 integrins to endothelium-expressed immunoglobulins such as ICAM-1 and VCAM-1 (Vestweber, 2015). In this way, the study of new compounds that can modulate some aspects of the inflammatory response and PMN migration is of interest in medicine.

Cell migration mechanisms are important points that were investigated in this study, demonstrating that delphinidin can attenuate cell migration. Additionally, *in vitro*, delphinidin attenuated the expression of CD11b and CD18 integrins after stimulation with LPS, showing yet another relevant anti-inflammatory action. The literature has demonstrated that delphinidin decreases the expression of α-IIbβ3 integrin activated on platelets, as well as the inhibition of platelet aggregation (Yang et al., 2012), and another study showed that delphinidin in colorectal cancer cells can downregulate integrin αV/β3 (Huang et al., 2019).

Moreover, as already mentioned, a reduction in the production of chemotactic cytokines such as CCL2 and IL-8 was observed. CCL2 stimulates chemotaxis and several cellular events, including calcium flux and integrin expression (Gschwandtner et al., 2019). Meanwhile, IL-

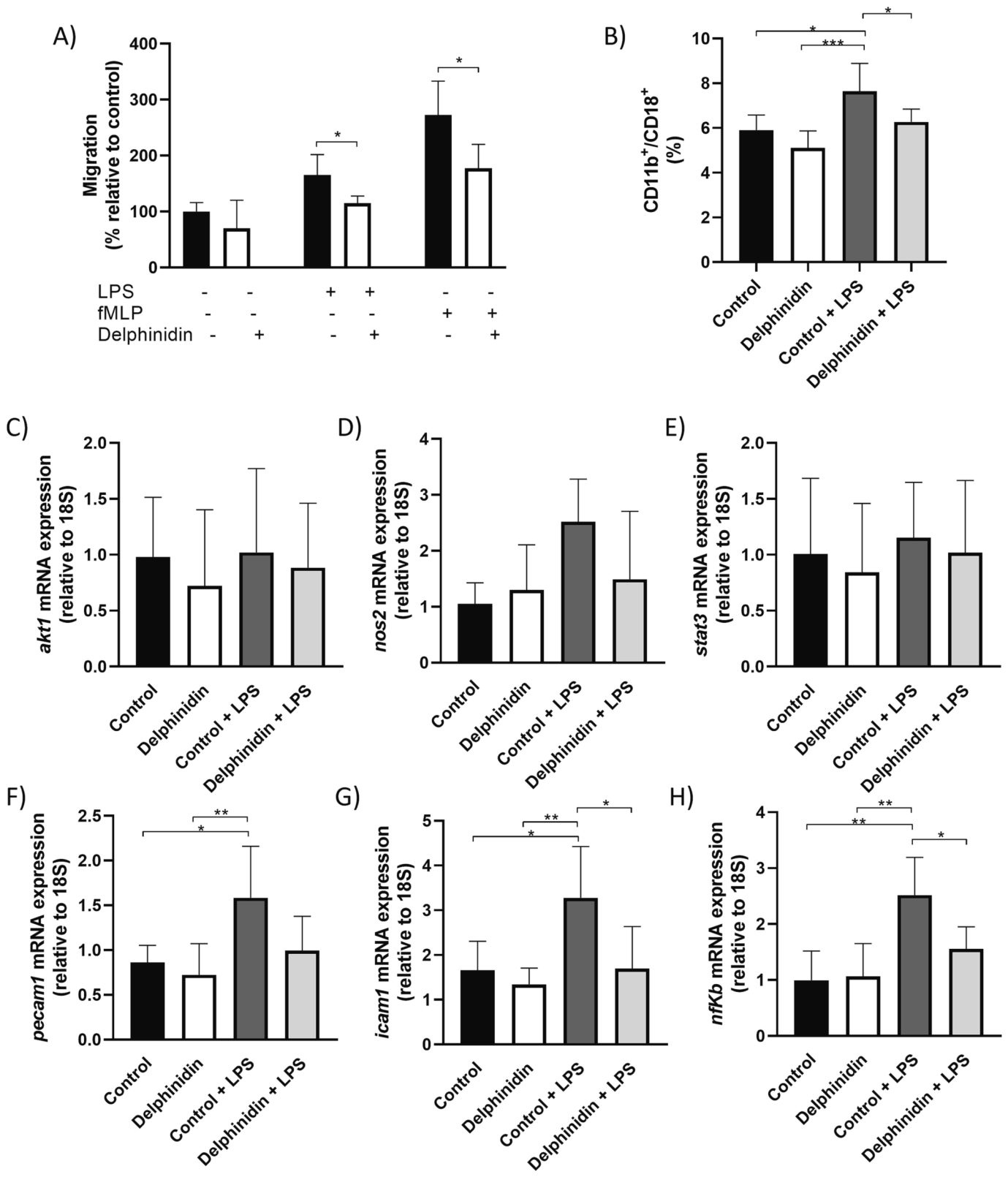

Fig. 3. The C1498 cells were cultivated with or without delphinidin and stimulated or not with LPS. Results of (A) cell migration (n = 4) (B) CD11/C14 positive cells expressions (n = 6) and gene expression (n = 6) of (C) *akt1*, (D) *nos2*, (E) *stat3*, (F) *pecam1*, (G) *icam1* and (H) *nfkb*. The results are expressed as the mean ± standard deviation. Asterisks indicate significant differences. *($p \leq 0.05$), **($p \leq 0.01$) and ***($p \leq 0.001$). *n* represents the number of samples evaluated.

8 is a potent chemotactic for neutrophils and a subset of T lymphocytes, inducing cytoskeletal reorganization, changes in intracellular calcium levels, integrin activation, and others (Matsushima et al., 2022).

Subsequently, in the *in vitro* experiments, a relevant result showed the action of delphinidin in reducing the transcription factor NFκB, a key point related to the inflammatory response. The anti-inflammatory activity of anthocyanins has been suggested by some authors (Joseph et al., 2016; Cremonini et al., 2022; Ma et al., 2021); however, despite the results found in the current study, showing more promising results *in vitro* than *in vivo*, some limitations of this study should be discussed. First, it can be challenging at times to compare results obtained *in vivo* and *in vitro* due to variations in experimental protocols. Second, from an ethical standpoint, we opted to utilize grape juice instead of pure delphinidin in our human study. Although we measured the delphinidin content in the grape juice, we are uncertain about its absorption rate or whether any metabolites might exert certain effects. Third, our study population was based on overweight women, and perhaps this population does not have an accentuated inflammatory process present to allow a more measurable attenuation due to grape juice intake. Finally, the reduced number of volunteers ensures sufficient power for statistical analysis. However, despite these limitations, some of the promising effects observed in the current study, related to the cell migration capacity, cannot be ruled out, and further studies are needed to improve our understanding.

In summary, the intervention of grape juice for a duration of 28 days was found to have an impact on certain properties associated with cell migration capacity. Moreover, *in vitro* experiments demonstrated that delphinidin can alter cell migration properties. These results provide novel information concerning the impact of grape juice and its compound delphinidin-3-O-glucoside on properties related to reducing cell migration capacity. Nevertheless, the underlying molecular mechanisms around this topic are not completely understood, and in the future, their underlying mechanisms of action should be explored.

CRediT authorship contribution statement

Juliana Gimenez Casagrande: Conceptualization, Methodology, Investigation, Data curation, Formal analysis. **Marcelo Macedo Rogero:** Conceptualization, Methodology, Writing – review & editing. **Dalila Cunha de Oliveira:** Methodology, Investigation, Data curation. **Bruna J. Quintanilha:** Methodology, Investigation, Data curation. **Vinícius Cooper Capetini:** Methodology, Investigation, Data curation. **Edson Naoto Makiyama:** Methodology, Investigation. **Bruna Roberta Oliveira Neves:** Methodology, Investigation. **Carlos Eduardo da Silva Gonçalves:** Methodology, Investigation, Methodology, Investigation. **Sumara de Freitas:** Investigation, Methodology. **Neuza Mariko Aymoto Hassimotto:** Methodology, Investigation. **Ricardo Ambrósio Fock:** Conceptualization, Methodology, Writing – original draft, Supervision, Project administration, Writing – review & editing, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113873.

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