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	intracellular oxidative stress. ConclusionsOur findings suggest the bread biofortified with selected durum wheat milling by-products as a source of phenolic acids with multiple anti-inflammatory and anti-atherosclerotic properties, which could help to counteract or prevent inflammatory vascular diseases.
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Phenolic extracts from whole wheat biofortified bread dampen overwhelming inflammatory response in human endothelial cells and monocytes: major role of VCAM-1 and CXCL-10

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Abstract

Purpose The aim of the study was to evaluate the vascular health action of extracts from biofortified bread, obtained by adding different durum wheat milling by-products rich in phenolic compounds, by analyzing their effects on overwhelming inflammatory response in endothelial cells and monocytes, two main players of atherogenesis.

Methods Human Umbilical Vein Endothelial Cells (HUVEC) or U937 monocytes were incubated with increasing concentrations (1, 5 or 10 μg/mL) of biofortified bread polyphenol extracts or corresponding pure phenolic acids before stimulation with lipopolysaccharide (LPS). We analyzed the endothelial-monocyte adhesion and related endothelial adhesion molecules expression. The expression of chemokines and pro-inflammatory cytokines were also measured in LPS-stimulated endothelial cells and monocytes as well as intracellular oxidative stress.

Results Biofortified bread extracts inhibited monocyte adhesion to LPS-stimulated endothelial cells, in a concentration dependent manner by reducing mainly endothelial VCAM-1 expression. Phenolic acid extracts contained in 10 mg biofortified bread down-regulated the LPS-induced expression of chemokines MCP-1, M-CSF and CXCL-10 as well as pro-inflammatory cytokines TNF-α and IL-1β, in endothelial cells and monocytes, with CXCL-10 as the most reduced inflammatory mediator. Among phenolic acids of biofortified bread, ferulic, sinapic and *p*-coumaric acids significantly inhibited the LPS-stimulated CXCL-10 expression in vascular cells. The reduced pro-inflammatory response was related to a slightly but significantly reduction of intracellular oxidative stress.

Conclusions Our findings suggest the bread biofortified with selected durum wheat milling by-products as a source of phenolic acids with multiple anti-inflammatory and anti-atherosclerotic properties, which could help to counteract or prevent inflammatory vascular diseases.

Keywords Human endothelial cells · Monocyte · Phenolic acids · Inflammation · Durum wheat milling by-products · Chemokine

Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall driven by innate and adaptive immune responses [1]. It is the underlying substrate of most cardiovascular diseases, which constitute the main cause of death in Western world and today also worldwide due to the increased prevalence of metabolic risk factors, including obesity and diabetes [2]. Atherosclerotic risk factors stimulate early activation and dysfunction of endothelial cells accompanied by the expression of adhesion molecules and chemokines, leading to sub-intimal infiltrations with mononuclear leukocytes (e.g., monocytes), the first morphological sign of vascular inflammation [3]. Once in the intima, leukocytes can be permanently activated by locally generated chemokines and cytokines, which can accelerate the transformation of monocytes/macrophages into foam cells and, on the other hand, could also induce the expression of endothelial adhesion molecules, including E-selectin, vascular cell adhesion molecule–1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), thus favoring the recruitment, adherence, and migration of leukocytes into inflamed vessel wall [4, 5].

Dietary patterns are major and modifiable factors in chronic inflammatory diseases [6]. Epidemiologic evidence indicates that whole-grain consumption substantially lowers the risk of cardiovascular disease, due to their unique health-promoting bioactive components of whole-grain, such as fiber and antioxidant phenolic compounds, mainly present in bran and germ fractions [7]. Dietary guidelines worldwide recommended increasing whole-grain consumption by replacing refined grains to counteract the prevalence of chronic diseases and cardio-metabolic risk factors [8-10]. Anyway, despite the public health recommendations, the consumption of whole grains is becoming very limited also in the Mediterranean countries [11], and is associated to a negative impact of most external bran layers on the sensorial parameters and quality of end-products. Recent advances in food technology have allowed the formulation of new functional whole wheat products with enhanced health-promoting value and safety without renouncing the good-tasting standards that are required by consumers. Previous researches have evaluated the bread-making ability of re-milled semolina from whole wheat, mixed with selected durum wheat milling by-products, obtained with new processing methodologies including debranning and micronization [12]. The technology of debranning, or pearling, is a dry separation technique consisting of progressive bran removal by consecutive abrasion of cereal kernels, which allows a separation of endosperm from the bran fraction ensuring the maximum concentration of desirable healthy phytochemicals in the bran [13]. These mechanical treatments allow the consecutive detachment of the outer, intermediate, and inner (the closest to the aleurone) layers of pericarp, leading to different by-product classes, namely the first, second, and third debranning fractions. In addition, these last two fractions can be mixed and submitted to dry fractionation by micronization and a subsequent air classification treatment, to give sub-fractions with different particle size that could increase the accessibility and probably also the bioavailability of bioactive compounds such as phenolic acids [14, 15]. A recent study reported that biofortified bread obtained by re-milled semolina enriched with 200 g/kg of milling by-products, usually destined to animal feed, as: i) residuals of the second and third debranning steps of durum wheat (DB), ii) the micronized and air-classified thin fraction obtained from the same residuals (MB), or iii) coarse bran obtained from conventional roller milling of non-debranned durum wheat (B), displayed increased antioxidant bioactive compounds, without negatively affecting bread physical properties in a significant way [16].

Given the positive chemical and sensory characteristics of these fortified breads, in the present study we investigated their potential anti-inflammatory nutraceutical properties. To this aim we used a well-known model of vascular inflammation and atherogenesis represented by cultured human endothelial cells and monocytes stimulated with bacterial lipopolysaccharide (LPS) [17, 18], a classical pro-inflammatory and pro-atherosclerotic agent. We monitored the nutraceutical effects of phenolic extracts from biofortified bread (B, DB, MB), by evaluating the monocyte adhesion to inflamed endothelial cells, the first obliged step of atherosclerosis development [19], as well as the related endothelial expression of adhesion molecules. Moreover, the gene expression of inflammatory mediators, including chemokines and pro-inflammatory cytokines was analysed in LPS-stimulated endothelial cells and monocytes.

Materials and Methods

Materials

The materials for cell cultures were obtained from EuroClone (Milan, Italy), CXCL-10 assay kit from R&D Systems (Minneapolis, MN, USA), primary human monoclonal antibodies against VCAM-1 from Merck Millipore (Milan, Italy) and peroxidase-conjugated secondary antibody from Santa Cruz Biotechnology (Dallas, Texas, USA). Unless otherwise indicated, all other reagents were purchased from Sigma-Aldrich (Milan, Italy). Re-milled semolina and milling by-products were obtained by Molini Tandoi S.p.A. (Corato, Italy), whereas fresh baker's yeast and food grade NaCl were purchased at local retailers.

Production of fortified breads by composite meals with re-milled semolina and selected durum wheat milling by-products

Re-milled semolina and three durum wheat (*Triticum durum*, Desf.) milling by-products were produced from the same grain lot at a local durum wheat milling industry (Molini Tandoi S.p.A., Corato, Italy). The by-products were: i) bran obtained by non-debranned wheat (B), ii) the second and third debranning fractions mixed together (DB), and iii) the thin sub-fraction obtained by micronization and air-classification of the second and third debranning fraction mix (MB). Processing technology, particle size and nutritional characteristics of by-products are described in a previous paper [12]. The by-products (200 g/kg) of B, DB, and MB were added to re-milled semolina to obtain a series of composed meals and then used to prepare fortified breads according to the bread-making procedure previously described [12]. Bread formulation included only fresh baker's yeast, NaCl and water, as traditionally made in Italy for durum wheat bread [20, 21], without using shortenings, malt, ascorbic acid, and potassium bromate.

Extraction of phenolic acids from ground freeze dried bread and HPLC analysis

Total phenolic acids (the sum of soluble and insoluble fractions) were extracted from the ground freeze dried bread samples following the method previously described by Laddomada et al., 2017 [22]. Prior to biological

assays in cells, the phenolic extracts were submitted to quali-quantitative analysis by HPLC according to the conditions reported in [22], showing the profile reported in Pasqualone et al., 2017 [16]. Briefly, the extracted compounds were quali-quantitatively analyzed using an Agilent 1100 Series HPLC-DAD system (Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed phase C18 (2) Luna column (Phenomenex, Torrance, CA, USA) (5 mm, 250 x 4.6 mm) at a column temperature of 30 °C. The wavelengths used for quantification of phenolic acids were 280, 295 and 320 nm. A mobile phase consisting of acetonitrile (A) and 10 mL/L water solution of H_3PO_4 (B) was used for the following elution program: isocratic elution, 100% B, 0-30 min; linear gradient from 100% B to 85% B, 30-55 min; linear gradient from 85% B to 50% B, 55-80 min; linear gradient from 50% B to 30% B, 80-82 min; and post time, 10 min before the next injection. The flow rate of the mobile phase was 1.0 mL/min, and the injection volume was 20 µL. Peaks identification was carried out by comparing their retention times and UV-Vis spectra to those of authentic phenolic standards. Individual phenolic acids were quantified via a ratio to the internal standard (3,5-dichloro-4-hydroxybenzoic acid) added to every sample and using calibration curves of phenolic acid standards.

Cell culture and treatment

Human umbilical vein endothelial cells (HUVEC) were isolated and grown in M199 medium containing 10% fetal bovine serum (FBS) as described [23]. Cells were obtained from discarded umbilical vein and treated anonymously conforming to the principles outlined in the Declaration of Helsinki. HUVEC were utilized up to the fifth passage from primary cultures. The human monocytic cell line U937 was purchased from the American Type Culture Collection (Rockville, MD) and grown in RPMI medium 1640 containing 10% FBS. U937 cell density was maintained at less than $1x10^6$ cells/ml to prevent cell differentiation. Phenolic acids extracts from biofortified bread (B, DB, MB) were dissolved in 70% ethanol and used after an appropriate dilution in culture medium. For treatment, confluent HUVEC or U937 cells were shifted to medium supplemented with 3% FBS for 4 h, and subsequently incubated in the absence or presence of phenolic extracts from biofortified bread at increasing concentrations (1, 5 or 10 µg/mL) or at 10 mg bread equivalent/mL for 2 h, before stimulation with inflammatory challenge lipopolysaccharide (LPS 0.5 µg/mL) for additional 4-20 h. In some experiments, HUVEC or U937 were incubated with pure phenolic acids (5 µg/mL) including ferulic acid (FRL), sinapic acid (SNP) or *p*-coumaric acid (CMR) for 2h before LPS stimulation. Stock solutions of phenolic acids were performed in 70% ethanol. As vehicle control, HUVEC or U937 were incubated with appropriate amount of ethanol (<0.025% v/v), which had no effect on any of the parameters measured, in our experimental conditions. Cellular toxicity by treatments was checked through a variety of techniques including Trypan blue exclusion assays and MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assays.

Monocytes endothelium adhesion assays

HUVEC were grown to confluence in 6-well tissue culture plates, incubated in the absence (vehicle) or presence of phenolic extract from biofortified bread (B, DB, MB) (1, 5, 10 µg/mL) for 2 h, and stimulated with LPS (0.5 µg/mL) for additional 16 h. U937 cells were labeled with 1 µmol/L calcein-AM (Molecular probe) for 30 min in RPMI medium 1640 containing 2.5 % FBS. In co-culture system, labeled U937 were seeded at 5x105 cell

density onto HUVEC monolayer and incubated under rotating conditions (63 rpm) at 21 °C, as described [17]. After washing, the adherent U937 cells were observed under fluorescence microscope by obtaining five photomicrographs from each well and then counted using the NHI Image analyzer program. Alternatively, the fluorescence intensity of each well was measured in a microplate reader with an excitation/emission wavelength of 485/530 nm.

Detection of endothelial cell surface molecules

HUVEC were grown to confluence in 96-well tissue culture plates, incubated in the absence (vehicle) or presence of phenolic extract from biofortified bread (B, DB, MB) (1, 5, 10 μg/mL) for 2 h, or, alternatively, with 10 mg bread equivalent/mL before stimulation with 0.5 μg/mL LPS for additional 6 h for E-Selectin or 16 h for ICAM-1 and VCAM-1. Assays of cell surface molecules were carried out by cell surface enzyme immunoassays (EIA) using primary mouse anti-human monoclonal antibodies against VCAM-1, ICAM-1 (HU5/3), E-selectin (Ab H18/7), or the monoclonal antibody against the non-cytokine-inducible and constitutive endothelial cell antigen E1/1, as previously described [23].

Chemokine release

HUVEC or U937 cells were treated with bread phenolic extracts (B, DB, MB) for 2 h at 10 mg bread equivalent/mL before stimulation with 0.5 μg/mL LPS for 24 h. The conditioned media were collected, and secreted CXCL-10 was determined in cell culture medium using the corresponding ELISA kits, according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction analysis

HUVEC or U937 cells were treated with bread phenolic extracts (B, DB, MB) for 2 h at 10 mg bread equivalent/mL or pure phenolic acids (FRL, SNP, CMR), before stimulation with 0.5 μg/mL LPS for 4 h. Total RNA was isolated by using the TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. For quantitative polymerase chain reaction, total RNA (2 μg) was converted into first-strand cDNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Monza, Italy). The quantitative RT-PCR (qRT-PCR) was performed in the Biorad Biosystems CFX384 Touch Real-Time PCR Detection System, by using SYBR Green PCR Master Mix. The gene cDNA fragments were amplified using primers synthesized by Sigma Genosys and reported in Table 1. The relative quantities of target gene mRNA against an internal controls, GAPDH mRNA, were measured by following a ΔCt method. The difference (ΔCt) between the main value in the triplicate samples of target gene and those of GAPDH mRNA were calculate using the CFX Manager (Version 3.0) Software (Biorad, Hercules, California USA), and the relative quantified value was expressed as $2^{-\Delta Ct}$.

Lipid peroxidation

The level of cellular lipid peroxidation was determined through the formation of thiobarbituric acid reactive species (TBARS) as reported in [24] following the method of Esterbauer and Cheeseman [25]. Briefly, malondialdehyde (MDA), a by-product of lipid peroxidation, forms an adduct with thiobarbituric acid (TBA) which was measured colorimetrically using an MDA equivalent standard. Butylated hydroxytoluene was added to each test sample to prevent further lipid oxidation during sample processing and the TBA reaction. The MDA production, expressed as nmoles produced/mg protein, was followed spectrophotometrically at 533 nm.

Statistical analysis

Results are expressed as means ± SD of at least 3 independent experiments. Differences between two groups were determined by unpaired Student's t test. Multiple comparisons were performed by one way analysis of variance (ANOVA), and individual differences then tested by the Fisher's protected least-significant difference test after the demonstration of significant inter-group differences by ANOVA. A p value < 0.05 was considered to be statistically significant.

Results

Biofortified bread polyphenolic extracts prevent monocyte adhesion to LPS-stimulated endothelial cells by reducing endothelial adhesion molecules expression

To evaluate the effects of biofortified bread polyphenol extracts on endothelial-monocyte adhesion, a crucial step in inflammatory and atherosclerotic process, HUVEC were pre-exposed to different phenolic acid extracts B, DB or MB at increasing concentrations (1, 5 or 10 μg/mL) before stimulation with LPS (0.5 μg/mL). Monocytoid cells did not adhere to unstimulated HUVEC monolayers (control, CTR) but strongly adhered to LPS-stimulated HUVEC (Fig. 1). The treatment of HUVEC with biofortified bread extracts significantly decreased LPS-induced monocyte adhesion in a concentration dependent fashion (Fig. 1), with an inhibitory effect already evident at 5 μg/mL. Greatest effect was observed at 10 μg/mL, inhibiting the monocyte adhesion to LPS challenged HUVEC by about 40%, 52% or 54% for B, DB or MB, respectively, as shown in bar graph (Fig. 1A) and in representative images (Fig. 1B). Noteworthy, the inhibitory effect of biofortified bread occurred only under pro-inflammatory conditions mimicked by LPS stimulation, without affecting the expression of the constitutive endothelial surface antigen E1/1. Moreover, polyphenolic extracts did not reduce endothelial cell viability as determined by MTT and Trypan blue assays (data not shown).

Since the adhesion of monocytes to endothelium is mediated by the increased expression of endothelial adhesion molecules, we investigated the effect of phenolic acids extracts of biofortified bread on the LPS-induced expression of VCAM-1, ICAM-1, and E-Selectin, by cell-surface EIA. The endothelial adhesion molecules were expressed at low levels in unstimulated HUVEC, and their expression was strongly boosted after LPS challenge (Fig. 2). All bread extracts affected the stimulated expression of endothelial adhesion molecules, mainly VCAM-1. As shown in Fig. 2 A, VCAM-1 expression was reduced in a concentration-dependent manner with significant effects already evident at 5 μg/mL, reaching at 10 μg/mL a reduction by about 42%, 45% or 52% for

B, DB or MB respectively (Fig. 2 A). An inhibitory trend was also evident for ICAM-1, achieving the statistical significance at 10 μg/mL for MB (Fig. 2B). Phenolic acid extracts significantly also reduced the cell surface expression of E-Selectin at 10 μg/mL (Fig. 2C).

To investigate the potential mechanism of action underlying the observed inhibitory effect on monocyte adhesion, we evaluated the endothelial expression of the gene coding for VCAM-1, being VCAM-1 the main endothelial adhesion molecule decreased by biofortified bread extracts. Quantitative real time PCR analysis revealed that polyphenolic extracts B, DB and MB reduced VCAM-1 mRNA levels in a concentration dependent fashion in LPS-stimulated endothelial cells (Fig. 3A), thus suggesting that the inhibitory action of the extracts occurred at pre-translation level. To compare the vasculo-protective action of different biofortified bread (B, DB, MB), we also analysed the effect of phenolic extracts obtained from the same amount of bread on VCAM-1 gene expression. We choose to investigate 10 mg biofortified bread, being the lowest quantity effective in reducing endothelial-monocyte adhesion. Indeed, as previously reported [16], 10 mg of B, DB and MB contained 5.7 ± 0.3, 6.5 ± 0.3 and 3.9 ± 0.2 μg phenolic acids, respectively, being ferulic acid the most abundant followed by sinapic acid and *p*-coumaric acid in all extracts [16]. The pre-treatment with phenolic extracts obtained from 10 mg of biofortified bread decreased significantly the mRNA levels of VCAM-1 in LPS-stimulated HUVEC, by about 21%, 23% or 28% for B, DB or MB respectively (Fig. 3B). These results confirmed the previous data on VCAM-1 reduction and showed that MB exhibited an inhibitory action similar to DB and B, despite a lower amount of total phenolic acids.

Biofortified bread extracts blunt endothelial inflammatory response

To deepen the vascular anti-inflammatory properties of phenolic acids from biofortified bread, we investigated the gene expression of endothelial inflammatory mediators such as chemokines and pro-inflammatory cytokines. LPS stimulation induced a strong inflammatory response in endothelial cells, raising the mRNA levels of the chemokines like monocyte chemoattractant protein-1 (MCP-1), macrophage colony-stimulating factor (M-CSF) and C-X-C motif ligand 10 (CXCL-10, also named interferon-inducible protein 10, IP-10) (Fig. 4A), as well as increasing the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Fig. 4B). In LPS-stimulated HUVEC, all biofortified bread extracts reduced the mRNA levels of chemokines and cytokines, although at different degree (Fig. 4). Among the endothelial inflammatory mediators, the chemokine CXCL-10 was the mostly significantly modulated by biofortified bread. Indeed, all extracts strongly inhibited the LPS-induced expression of CXCL-10 by about 50%. They also significantly decreased the stimulated mRNA levels of M-CSF (Fig. 4A), as well as TNF-α and IL-1β (Fig. 4B), although to a lesser extent; whereas the inhibition of MCP-1 mRNA levels did not reach statistical significance (Fig. 4A).

Biofortified bread extracts attenuate the overwhelming inflammatory response in LPS stimulated monocytes

To assess whether the anti-inflammatory action of biofortified bread occurred also in other vascular cells, different from endothelium, we investigated the effects of extracts on human monocytic U937 cells activated by LPS challenge, by measuring the mRNA levels of chemokines and pro-inflammatory cytokines. LPS stimulation strongly increased the monocyte gene expression of MCP-1, M-CSF, CXCL-10, TNF-α and IL-1β

(Fig. 5). Biofortified bread extracts B, DB, MB significantly blunted the over-expression of all analyzed chemokines and pro-inflammatory cytokines in monocytes, with a greater inhibitory effect than in endothelium. As in endothelial cells, also in monocytes CXCL-10 resulted the inflammatory mediator mostly reduced by bread extracts, reaching an inhibition by about 65%, 72% or 69% for B, DB or MB respectively (Fig. 5). The anti-inflammatory effect of biofortified bread was not associated with cytotoxic action of extracts in monocytes, since they did not modify cell viability, as determined by MTT and Trypan blue assays (data not shown).

Phenolic acids from biofortified bread inhibit CXCL-10 expression in inflamed endothelial cells and monocytes

Since biofortified bread greatly decreased CXCL-10 expression, we confirmed their inhibitory effect at the protein level in endothelial cells and monocytes. LPS stimulation strongly induced CXCL-10 protein release both in HUVEC or in U937 by about 4-fold or 16-fold over unstimulated controls, respectively (Fig. 6). Biofortified bread extracts significantly reduced the CXCL-10 protein release in LPS stimulated endothelial cells lowering it to the value of unstimulated control. Analogous results were reported in U937, where CXCL-10 protein was strongly lowered by all analyzed extracts (74% for B, 75% for DB and 78% for MB). In an attempt to analyze the contribution of specific phenolic acids present in biofortified bread extracts, we evaluated the effect on CXCL-10 expression by main phenolic acids present in biofortified bread extracts including ferulic acid, sinapic acid and *p*-coumaric acid. To this aim, before stimulation with LPS, HUVEC and U937 were pre-treated with 5 μg/mL phenolic acids, corresponding to the lowest concentration of bread extracts able to reduce the adhesion of leukocytes to inflamed endothelial cells. As shown in Figure 7, all pure phenolic acids significantly inhibited the LPS-stimulated expression of CXCL-10, in endothelial cells and monocytes (Fig. 7). The inhibition rank by ferulic, sinapic and *p*-coumaric acid was lower than that observed with biofortified bread extracts, thus suggesting the greater anti-inflammatory effectiveness of bread extracts, probably due to a synergism among phenolic acids.

Phenolic extracts from biofortified bread reduced oxidative stress in LPS challenged vascular cells

In vascular cells, it has been recognized that LPS induced inflammatory response occurred through an overproduction of intracellular reactive oxygen species (ROS) [17, 18, 26], which could induce direct damages of cellular lipids, the so-called lipid peroxidation [27]. Since we have previously shown that phenolic extracts from biofortified bread exhibited antioxidant potential in cell free-systems [12], here we decided to evaluate the effects of bread extracts or corresponding pure phenolic acids on LPS-induced lipid peroxidation in endothelial cells and monocytes, as revealed by level of its end product MDA [27]. Fig. 8 shows that LPS stimulation produced an increased lipid peroxidation, as assayed by MDA content, both in HUVEC (Fig. 8A) and U937 (Fig. 8B). The pre-exposure with B, DB, MB significantly decreased the MDA levels induced by LPS stimulation. Similar to bread extracts, pure phenolic acids, including ferulic, sinapic and *p*-coumaric acids, lowered the MDA content to a similar extent.

345 **Discussion**
346
347 Cereal-based products are the most common staples in the world, and cereal products like whole wheat
348 bread can represent an attractive functional food and a vehicle for bioactive compounds, including phenolic
349 acids. In this study, we characterized the nutraceutical anti-inflammatory properties of functional bread
350 biofortified with selected durum wheat milling by-products rich in phenolic compounds, by using an *in vitro*
351 model of inflammation and atherogenesis.
352 We found that phenolic extracts obtained from different biofortified bread (B, DB and MB) inhibited, in a
353 concentration-dependent manner, the adhesion of monocyte to inflamed endothelium. Recruitment of monocytes
354 from the circulation, their adhesion to endothelial cells and subsequent trans-endothelial migration into the
355 intima of vascular wall constitute the initial steps of atherosclerosis development [19]. These interactions
356 between monocytes and endothelial cells involve cell adhesion molecules, such as E-Selectin, VCAM-1 and
357 ICAM-1 on the surface of the activated endothelium [28]. We found that phenolic acids from biofortified bread
358 inhibited endothelial-monocyte adhesion by preventing the related expression of leukocyte adhesion molecules,
359 particularly VCAM-1, which recognizes and binds the VLA4 (Very Late Antigen-4) counter-receptor on
360 monocytes and lymphocytes, causing their adhesion at the site of activation. Once adherent to the endothelium,
361 leukocytes migrate into the underlying intima in response to chemoattractant stimuli including chemokines.
362 Several categories of chemokines may participate in recruitment of distinct leukocyte classes to the
363 atherosclerotic lesions including MCP-1, which attracts leukocytes bearing the chemokine receptor CCR2, i.e.,
364 monocytes and T and B cells, as well as the monokine CXCL-10 also involved in leukocyte recruitment, all
365 chemokines found in human and experimental lesions [29]. The growth factor M-CSF also contributes to the
366 monocyte migration into the artery wall, and can induce endothelial cell-derived MCP-1. We found that
367 biofortified bread extracts down-regulated the gene expression of chemotactic cytokines, including M-CSF and
368 CXCL-10 in LPS triggered endothelial cells. Even more marked inhibitory effects were observed on
369 overwhelming inflammatory response of monocytes, where bread extracts significantly reduced mRNA of M-
370 CSF, CXCL-10 as well as MCP-1, thus highlighting a multiple interference to counteract the leukocyte
371 recruitment in the vessel wall. Other pivotal pro-inflammatory cytokines, like TNF-α and IL-1β, can promote
372 leukocyte adherence and migration, resulting in a worsening of endothelial dysfunction [30]. Phenolic acids
373 extracts decreased the gene expression of TNF-α and IL-1β, in vascular cells, thus amplifying the anti-
374 inflammatory response by biofortified bread. Our findings are in agreement with a previous nutritional
375 intervention trial showing that whole-grain wheat consumption reduced systemic inflammation lowering TNF-α
376 levels in overweight and obese subjects [10], and reveal endothelial cells and monocytes as possible cellular
377 actors involved in the reduced overwhelming inflammatory response by whole grain. The nutraceutical activity
378 of whole wheat biofortified bread (B, DB, MB), investigated in this paper, can be at least partially explained by
379 the anti-inflammatory properties of ferulic acids, the most abundant phenolic acid in our extracts and in whole
380 grain [31, 32]. Indeed, previous studies *in vitro* and in animal models showed that ferulic acid inhibited ICAM-
381 1, VCAM-1 and E-selectin expression and leukocyte adhesion to the endothelium [33, 34], as well as attenuated
382 inflammation and oxidative stress in a rat model of LPS-induced acute respiratory distress syndrome [35].
383 Moreover, sinapic and *p*-coumaric acids also can contribute to the anti-inflammatory activity of biofortified

384 bread extracts, since they have been shown to reduce endothelial adhesion molecules, the inflammatory
385 cytokines IL-6, TNF-α and IL-1β, as well as the MCP-1 and M-CSF [17, 31, 32, 36, 37].
386 In this study, we provided new evidence of the anti-inflammatory effects of biofortified bread extracts
387 which were able to reduce the LPS-induced expression and release of CXCL-10 both in activated endothelium
388 and monocytes. Moreover, we showed for the first time, to the best of our knowledge, that phenolic acids such
389 as ferulic, sinapic and *p*-coumaric acid, reduced the expression of CXCL-10 in endothelial cells and in
390 monocytes under stimulation with the inflammatory trigger, LPS. However, the different phenolic acids, taken
391 individually, showed lower anti-inflammatory efficacy than biofortified bread extracts; indeed, ferulic acid, the
392 most abundant phenolic acid in biofortified bread, reduced CXCL-10 expression by about 20% or 33% in
393 endothelium or monocytes respectively, against the reduction by about over 50% in HUVEC and 65% in U937
394 by corresponding biofortified bread extracts. These findings suggest a synergistic action among different
395 phenolic acids of extracts in reducing CXCL-10, which can result in blood vessels protection against chronic
396 inflammation. In many inflammatory diseases, CXCL-10 is thought to play an important role in recruiting
397 activated T cells into sites of tissue inflammation [38]. Moreover, CXCL10 is highly expressed in human
398 atheroma throughout all stages of plaque development, and serum CXCL-10 levels are associated with the
399 severity of coronary artery disease and coronary artery occlusion [39]. Genetic absence of CXCL-10 conferred a
400 reduction in early aortic lesion formation with a marked reduction in T cells positive for CXCR3, the receptor of
401 CXCL-10, as well as CXCL-10 neutralization can ameliorate LPS-induced acute respiratory distress syndrome
402 in rats [40]. It is well recognized that oxidative stress mediates the LPS-induced proinflammatory response [18].
403 We showed that the anti-inflammatory action of biofortified bread extracts was associated with reduced
404 intracellular oxidative stress, consistent with decreased levels of lipid peroxidation. These findings
405 demonstrating that whole grain bread extracts exhibited antioxidant action in LPS-challenged vascular cells,
406 confirm and extend their antioxidant properties revealed in cell-free systems [12]. They are in agreement with
407 reduced intracellular ROS observed in LPS-challenged human endothelial progenitor cells by fermented whole
408 grain [18]. Phenolic acids, including ferulic, sinapic and *p*-coumaric acids, contribute to the antioxidant activity
409 of the extract, reducing the intracellular oxidative stress to a similar extent of bread extracts. Our results are in
410 accordance with previous studies showing that the anti-inflammatory action of phenolic acids, including ferulic,
411 sinapic and *p*-coumaric acids, was mediated by lowered intracellular ROS levels associated with reduced
412 activation of NF-kB, a pivotal redox sensitive transcription factor, involved in the regulation of inflammatory
413 gene expression [17, 41, 42].
414 Overall, by comparison the nutraceutical properties of phenolic acid extracts of different fortified
415 bread, we found that the breads obtained with the addition of 20% of second and third decortication, without or
416 with micronization, named DB and MB, respectively, preserve and even improve the nutraceutical properties of
417 the whole wheat bread obtained with 20% of bran (B), suggesting that the fortified breads MB and DB can be
418 considered as functional breads improving vascular function and providing health protection in addition to
419 ensure quality and palatability [12]. They inhibit endothelium-monocyte adhesion and endothelial cell activation
420 through downregulation of adhesion molecules, chemoattractants and pro-inflammatory cytokines (Fig. 9).
421 These last two classes of inflammatory mediators were also decreased in monocytes stimulated with LPS,
422 highlighting a role of the phenolic acids of the bread in counteracting leukocyte activation, an important
423 phenomenon in atherosclerosis and in chronic inflammatory pathologies.

Our findings being derived from *in vitro* models could not be directly extrapolated to humans. However, they can be biologically relevant, since the use of whole grain processed materials in substitution of raw materials could improve the bio-accessibility and therefore can also influence the bioavailability of the phenolic content *in vivo* [43]. Moreover, since in whole grain, about 95% of phenolic acids are covalently bound to cell wall polysaccharides through ester bonds, whole grain fiber can deliver phenolic compounds into the lower gut, and the slow and continuous release of ferulic acid by the action of gut microbiota metabolism can increase circulating ferulic acid and its metabolites, thus possibly providing an amelioration of chronic inflammation and the long term benefits associated with whole grain consumption [10].

In conclusion, our findings provide evidence suggesting the whole wheat bread biofortified with selected durum wheat milling by-products as a source of phenolic acids with multiple healthful biological properties, which occur through a nutrigenomic mechanism via modulation of the expression of genes involved in monocyte adhesion, trans-endothelial migration and immune response.

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Conflict of Interest The authors declare that they have no conflict of interest.

Figure captions

Fig. 1 Inhibitory effects of phenolic acid extracts from biofortified bread on monocyte adhesion to LPS stimulated endothelial cells. HUVEC were pre-treated with B, DB or MB phenolic extracts (1, 5, 10 μg/mL) or vehicle (control, CTR) for 2 h and then stimulated with LPS 0.5 μg/mL for 16 h. Then, HUVEC were co-cultured with calcein AM-labeled U937 monocytes. The number of adherent U937 cells was measured by fluorescence plate reader (A) or monitored by fluorescence microscope (B). The values of monocyte adhesion are represented in comparison to monocyte adhesion to LPS-stimulated HUVECs, normalized at 100% (A). In B, representative images of endothelial-monocyte adhesion after pre-incubation with phenolic extracts (10 μg/mL). Each experiment was performed in triplicate. #p < 0.01 *vs* control; **p* < 0.05; ***p* < 0.01 *vs* LPS alone.

Fig. 2 Concentration-dependent effect of phenolic acids from biofortified bread on the surface expression of endothelial adhesion molecules. HUVEC were pre-incubated with B, DB or MB phenolic extracts (1, 5, 10 μg/mL) or vehicle (control, CTR) for 2 h and then stimulated with LPS 0.5 μg/mL. Cell surface expression of VCAM-1 (A), ICAM-1 (B), and E-Selectin (C) was analysed by cell surface EIA. Each experiment was performed in triplicate. Data are expressed as the percentage of LPS-induced expression (mean ± S.D.). #*p* < 0.01 *vs* control; **p* < 0.05; ***p* < 0.01 *vs* LPS alone.

Fig. 3. Effects of bread extracts on LPS stimulated mRNA levels of VCAM-1 in endothelial cells. HUVEC were pre-incubated with phenolic extracts at 1, 5, 10 μg/mL (A) or 10 mg bread eq/mL (B) for 2 h before stimulation with 0.5 μg/mL LPS for further 4 h, then mRNA levels of VCAM-1 were determined by quantitative RT-PCR. Data are representative of four independent experiments (mean ± SD) and expressed as percentage of LPS-stimulated endothelial cells. #*p* < 0.05 vs CTR; **p* < 0.05 *vs* LPS alone.

Fig. 4. Effects of phenolic acids extracts from biofortified bread on endothelial expression of inflammatory markers. HUVEC were pre-treated with B, DB or MB (10 mg bread eq/mL) or vehicle (CTR) for 2 h and then stimulated with LPS 0.5 μg/mL for 4 h. mRNA levels of MCP-1, M-CSF and CXCL-10 (A) as well as TNF-α and IL-1β (B) were determined by quantitative RT-PCR. Data are representative of three independent experiments (mean ± SD), each performed in triplicate, and expressed as percentage of LPS-stimulated endothelial cells. #*p* < 0.05 vs CTR; **p* < 0.05 *vs* LPS alone.

Fig. 5. Effects of phenolic acids extracts from biofortified bread on monocytic expression of inflammatory markers. U937 were pre-treated with B, DB or MB (10 mg bread eq/mL) or vehicle (CTR) for 2 h and then stimulated with LPS 0.5 μg/mL for 4 h. mRNA levels of MCP-1, M-CSF and CXCL-10 (A) as well as TNF-α and IL-1β (B) were determined by quantitative RT-PCR. Data are representative of three independent experiments (mean ± SD), each performed in triplicate, and expressed as percentage of LPS-stimulated endothelial cells. #*p* < 0.05 *vs* CTR; **p* < 0.05 *vs* LPS alone.

Fig. 6 Inhibitory effects of phenolic extracts from biofortified bread on CXCL-10 protein release in LPS-stimulated HUVEC or U937 monocytic cells. HUVEC or U937 were pre-treated with B, DB or MB (10 mg bread eq/mL) or vehicle (CTR) for 2 h and then stimulated with LPS 0.5 μg/mL for 18 h. CXCL-10 protein release was determined in culture medium by ELISA. Results are reported as percentage of LPS-induced expression (mean ± S.D.). Data are representative of three independent experiments. #$p < 0.01$ *vs* CTR; *$p < 0.01$ *vs* LPS alone.

Fig. 7 Inhibitory effects of pure phenolic acids on CXCL-10 expression in endothelial cells and monocytes. HUVEC or U937 were pre-treated with 5 μg/mL pure phenolic acids (FRL, SNP, CMR) for 2 h and then stimulated with LPS (0.5 μg/mL) for further 4 h, after which CXCL-10 mRNA levels were determined by quantitative RT-PCR. Data are representative of three independent experiments (mean ± SD), each performed in triplicate, and expressed as percentage of LPS-stimulated endothelial cells. #$p < 0.05$ *vs* CTR; *$p < 0.05$ *vs* LPS alone.

Fig. 8 Effects of B, DB and MB extracts or pure phenolic acids on LPS-induced oxidative stress in endothelial cells and monocytes. HUVEC or U937 were pre-treated with B, DB or MB (10 mg bread eq/mL) or vehicle (CTR) for 2 h and then stimulated with LPS 0.5 μg/mL for 16 h, after which lipid peroxidation was evaluated by MDA content. Data are representative of three independent experiments (mean ± SD) and expressed as percentage of LPS-stimulated cells. #$p < 0$ 05 *vs* CTR; *$p < 0.05$, *vs* LPS alone.

Fig. 9 Synthetic picture about the anti-inflammatory properties of polyphenolic extracts from whole wheat biofortified bread in LPS stimulated endothelial cells and monocytes.

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638

Figure 1

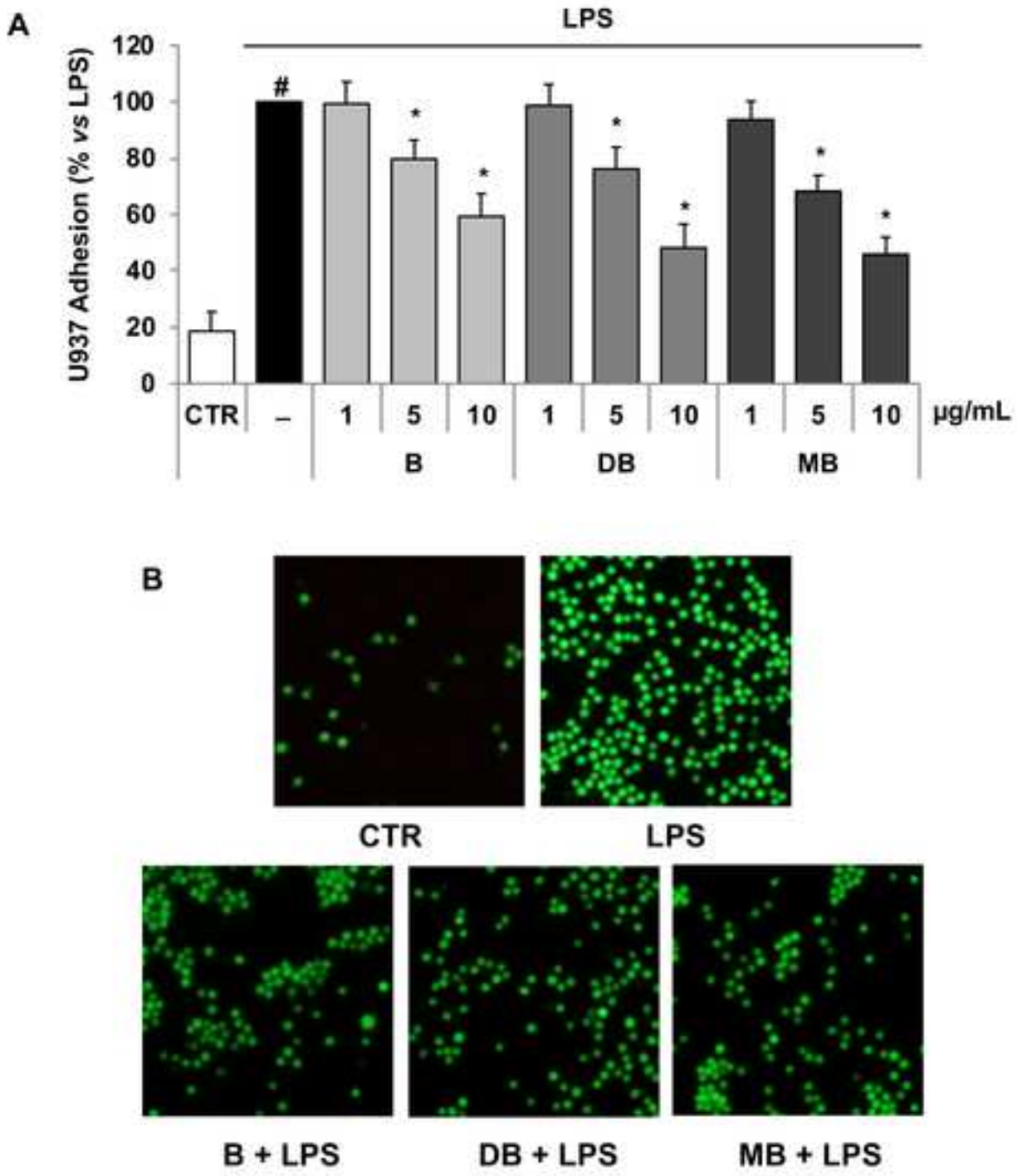

Fig. 1

Figure 2

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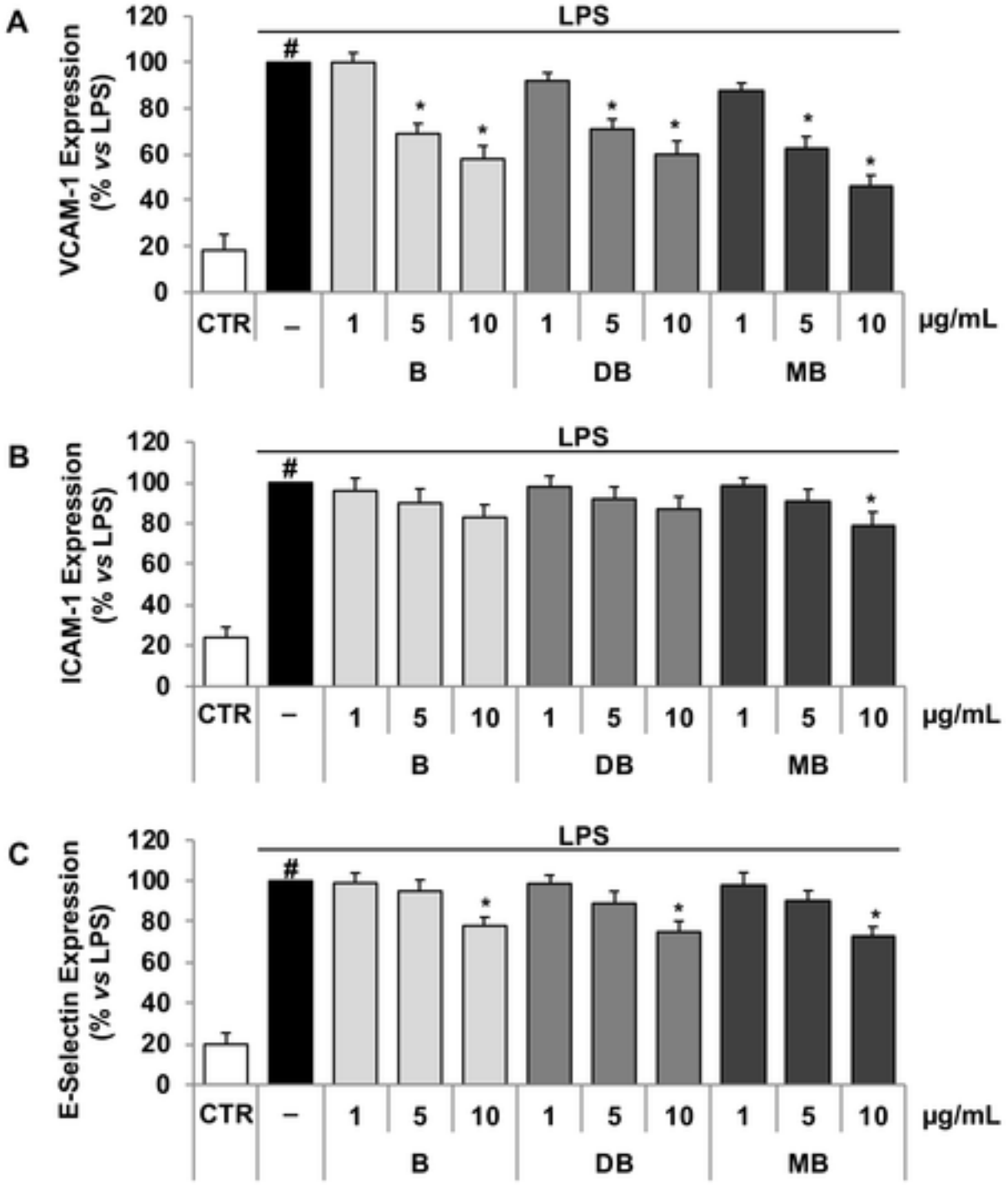

Fig. 2

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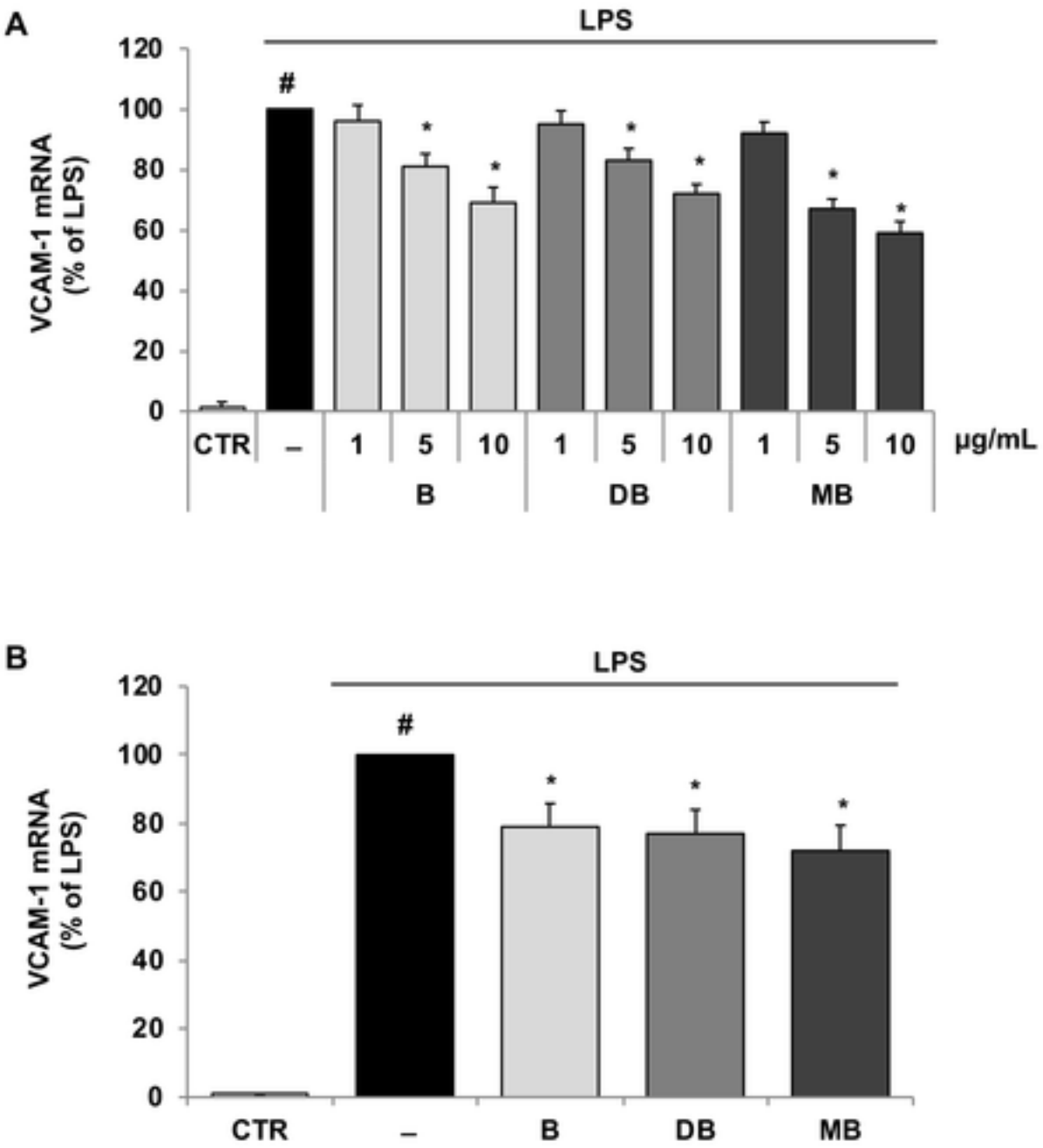

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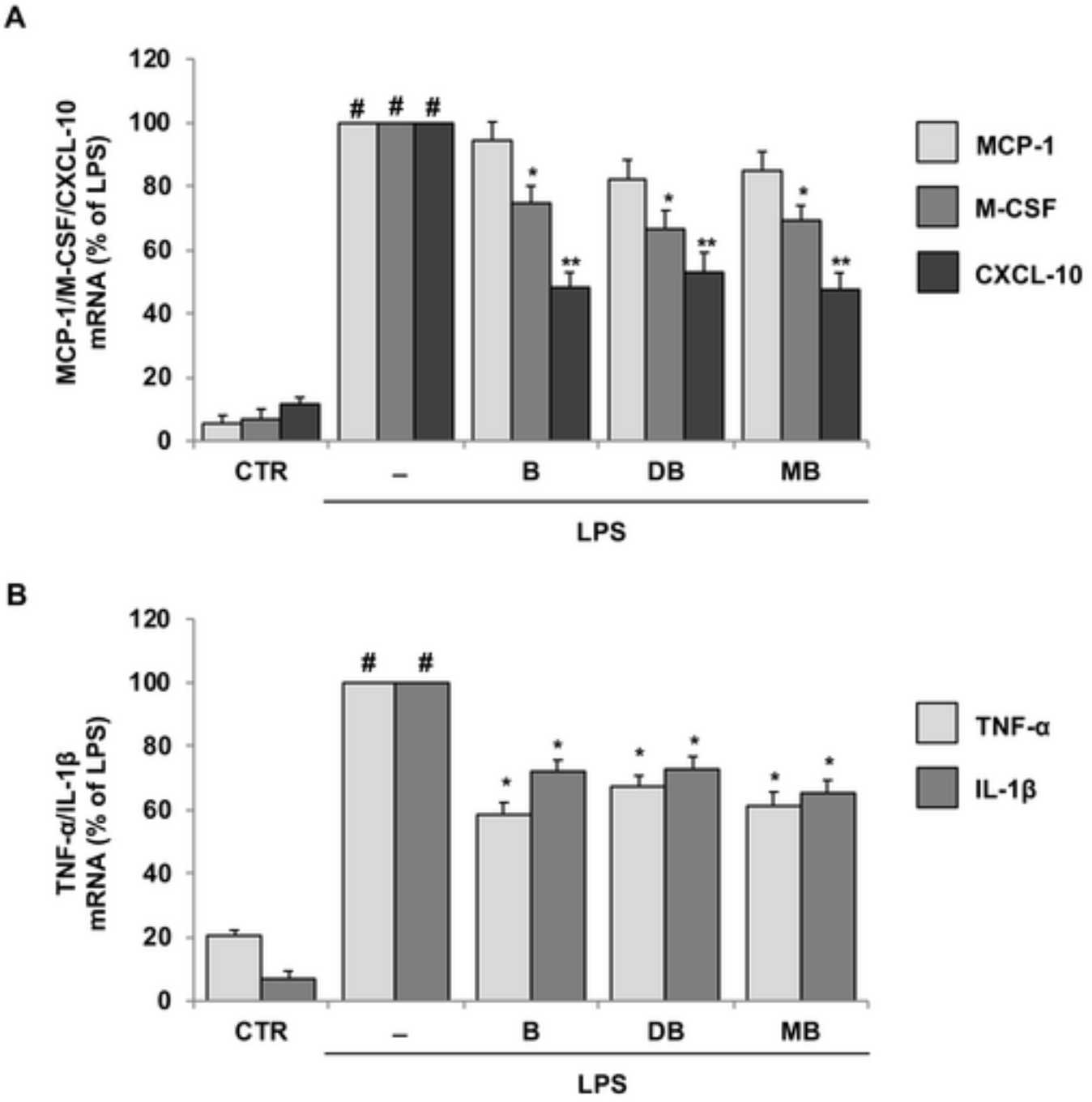

Fig. 4

Figure 5

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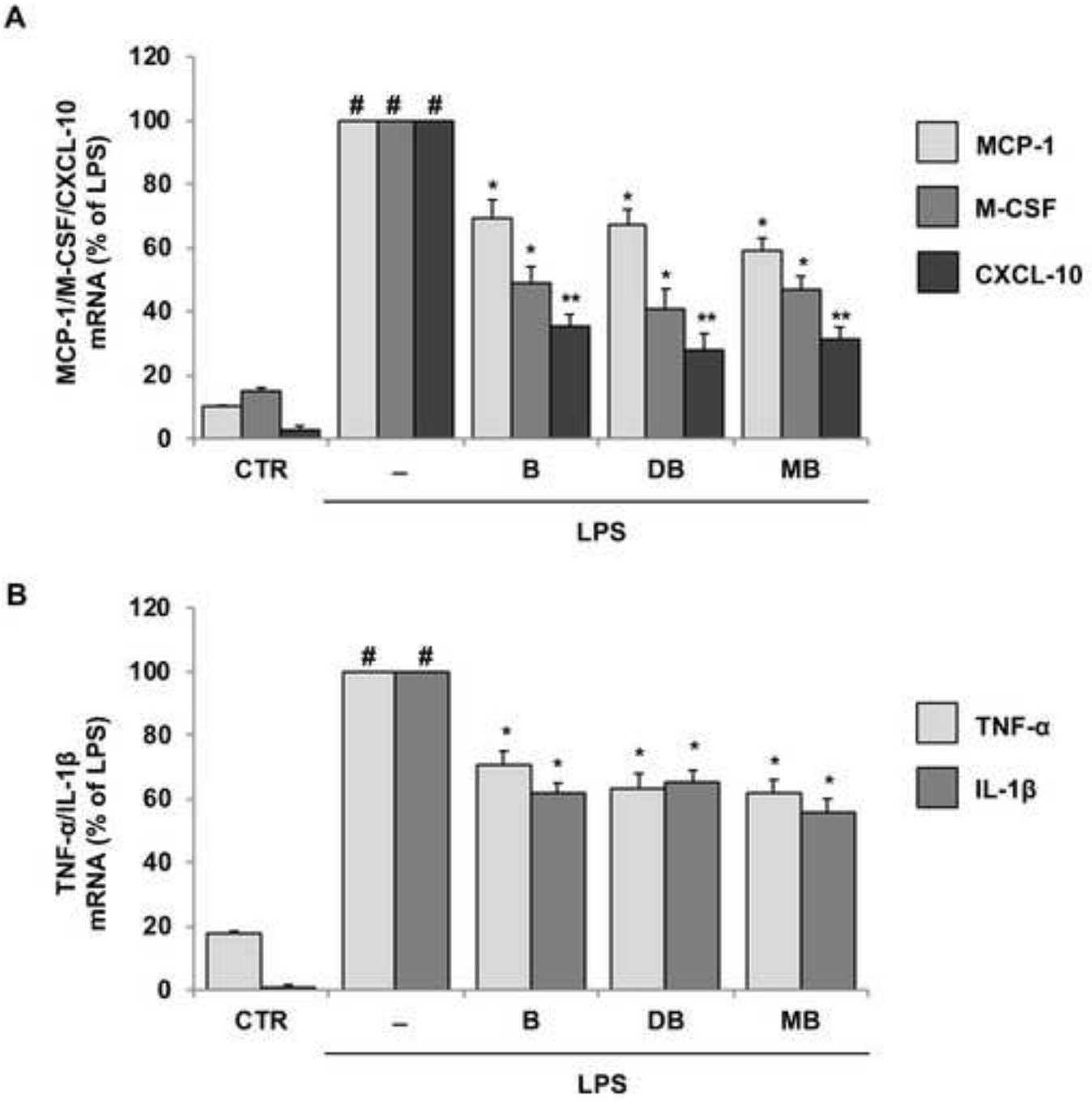

Fig. 5

Figure 6

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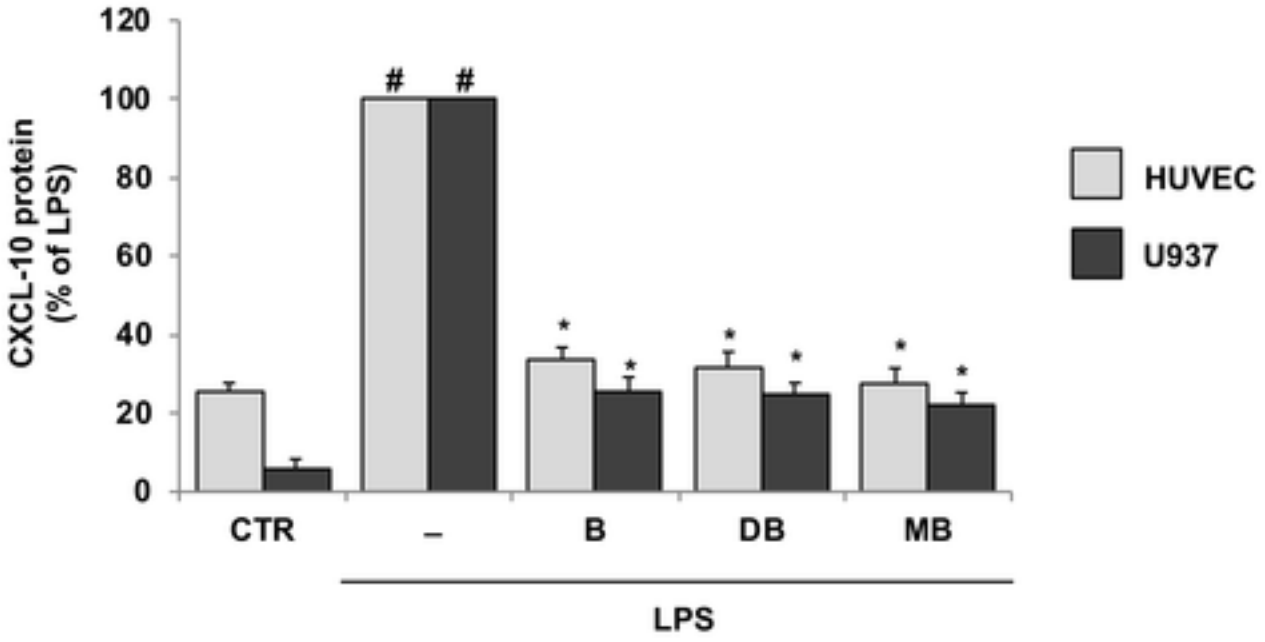

Fig. 6

Figure 7

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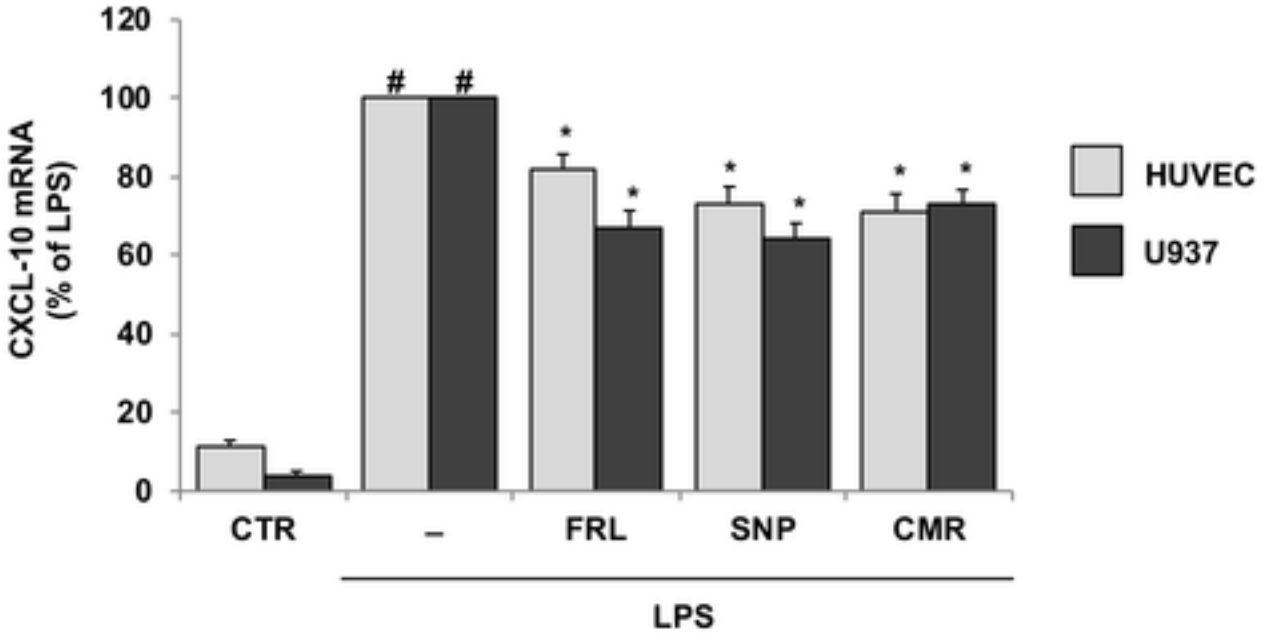

Fig. 7

Figure 8

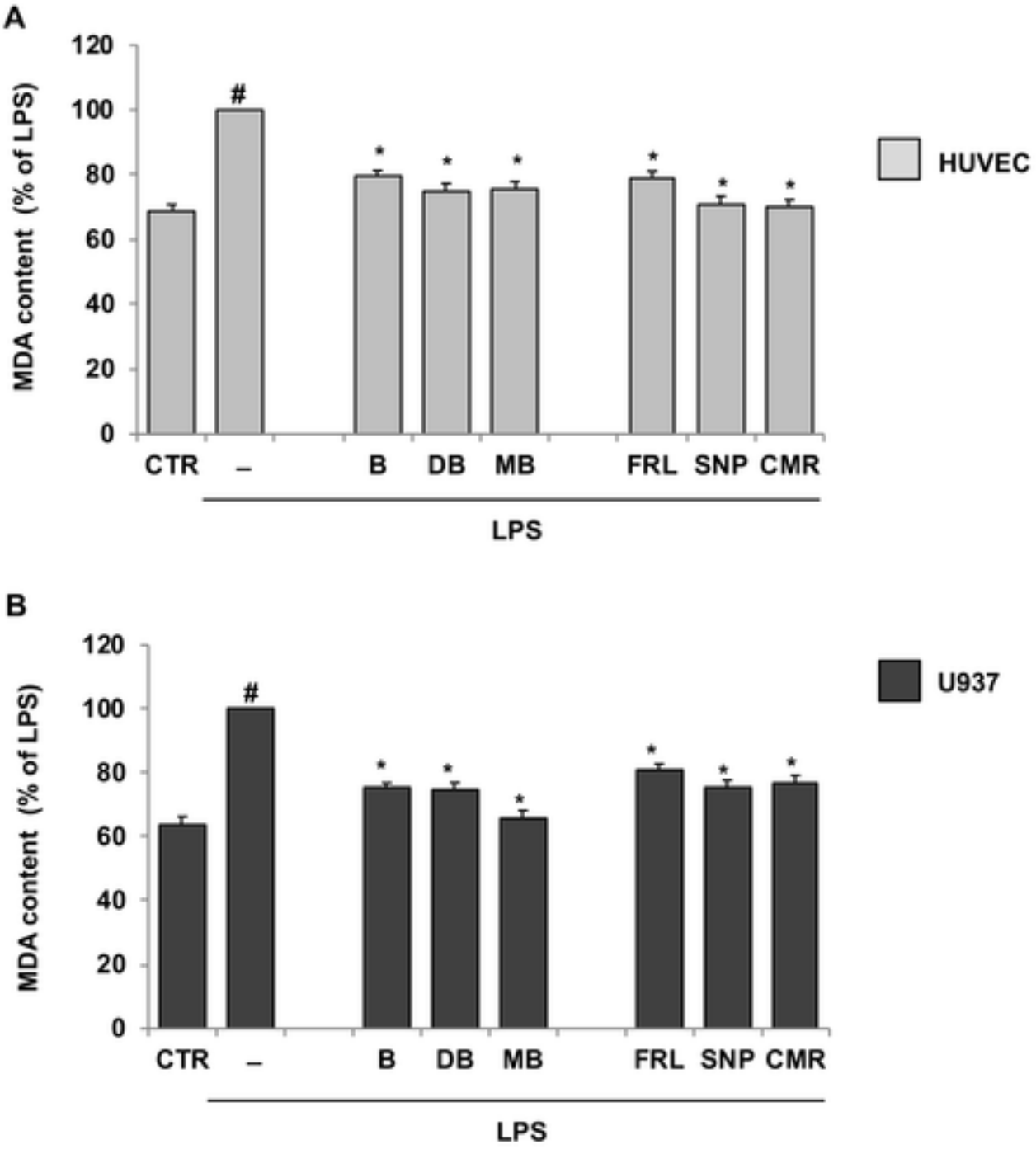

Fig. 8

Figure 9

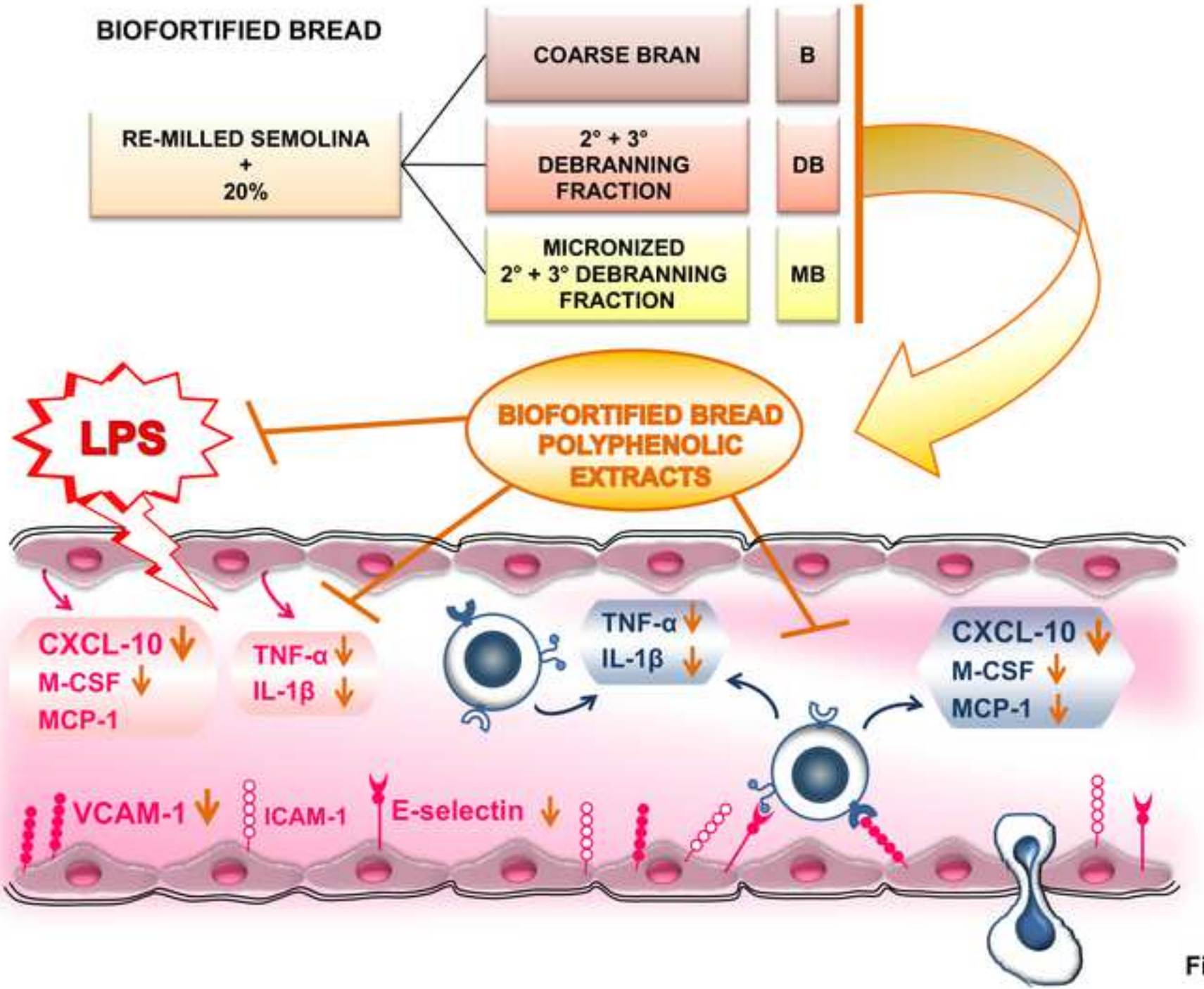

Fig. 9

Tables

Table 1 Primers sequences of real time quantitative PCR

GENE	ACCESSION NUMBER	PRIMERS (sequence 5'-3')	SIZE (bp)
TNF-α	NM_000594.2	CCTGTGAGGAGGACGAACAT AGGCCCCAGTTTGAATTCTT	240
IL-1β	NM_000576.2	CTGTCCTGCGTGTTGAAAGA AGTTATATCCTGGCCGCCTT	228
VCAM-1	NM_00107B.3	CATGGAATTCGAACCCAAAC CCTGGCTCAAGCATGTCATA	140
ICAM-1	NM_000201.2	AGACATAGCCCCACCATGAG CAAGGGTTGGGGTCAGTAGA	190
E-Selectin	NM_000450.2	ATGTGAAGCTGTGAGATGCG CCACTGCAGCTCATGTTGAT	252
MCP-1	NM_002982.3	CCCCAGTCACCTGCTGTTAT CCTGAACCCACTTCTGCTT	166
M-CSF	NM_000757.4	TGGACGCACAGAACAGTCTC CCTCCAGGGCTCACAATAAA	235
CXCL-10	NM_001565.2	CAAGGATGGACCACACAGAG GCAGGGTCAGAACATCCACT	248
GAPDH	NG_007073.2	ATGCCTTCTTGCCTCTTGTC CATGGGTGGAATCATATTGG	245