

Multiple anti-inflammatory and anti-atherosclerotic properties of red wine polyphenolic extracts: differential role of hydroxycinnamic acids, flavonols and stilbenes on endothelial inflammatory gene expression

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Abstract

Purpose The aim of the study was to evaluate the vascular anti-inflammatory effects of polyphenolic extracts from two typical South Italy red wines, the specific contribution of individual polyphenols and the underlying mechanisms of action.

Methods Human endothelial cells were incubated with increasing concentrations (1–50 µg/mL) of Primitivo and Negroamaro polyphenolic extracts (PWPE and NWPE, respectively) or pure polyphenols (1–25 µmol/L), including hydroxycinnamic acids (*p*-coumaric, caffeic and caffeic acids), flavonols (kaempferol, quercetin, myricetin) or stilbenes (*trans*-resveratrol, *trans*-piceid) before stimulation with lipopolysaccharide. Through multiple assays, we analyzed the endothelial–monocyte adhesion, the endothelial expression of adhesion molecules (ICAM-1, VCAM-1 and E-Selectin), monocyte chemoattractant protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF), as well as ROS intracellular levels and the activation of NF-κB and AP-1.

Results Both PWPE and NWPE, already at 1 µg/mL, inhibited monocyte adhesion to stimulated endothelial

cells, a key event in triggering vascular inflammation. They down-regulated the expression of adhesion molecules, ICAM-1, VCAM-1, E-Selectin, as well as MCP-1 and M-CSF, at mRNA and protein levels. All polyphenols reduced intracellular ROS, and everything, except caffeic acid, inhibited the endothelial expression of adhesion molecules and MCP-1, although with different potency. Flavonols and resveratrol significantly reduced also the endothelial expression and release of M-CSF. The decrease in endothelial inflammatory gene expression was related to the inhibition of NF-κB and AP-1 activation but not to intracellular oxidative stress.

Conclusions This study showed multiple anti-inflammatory and anti-atherosclerotic properties of red wine polyphenolic extracts and identified specific bioactive polyphenols which could counteract inflammatory diseases including atherosclerosis.

Keywords Macrophage colony-stimulating factor · Monocyte chemoattractant protein-1 · Vascular cell adhesion molecule-1 · Intercellular adhesion molecule-1 · E-Selectin

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Introduction

Atherosclerosis is a chronic, progressive disease of the arterial wall with relevant inflammatory components in its inception, progression and complications [1, 2]. Qualitatively and quantitatively abnormal atherosclerotic stimuli, likely through the induction of heightened oxidative stress, alter the normal homeostatic function of the vascular endothelium and cause endothelial activation. It consists in a concerted transcriptional activation of genes involved in recruitment of leukocytes, mainly monocytes, into the

intima, including genes encoding for leukocyte adhesion molecules such as E-Selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1); chemotactic factors such as monocyte chemoattractant protein-1 (MCP-1); and growth factors such as macrophage colony-stimulating factor (M-CSF) [3]. Adhesion molecules, chemoattractants and growth factors are over-expressed in atherosclerotic lesions and involved in the focal recruitment of monocytes [1]. The first step in monocyte adhesion is mediated by selectins, the firm adhesion by immunoglobulins ICAM-1 and VCAM-1, and the subsequent transmigration and differentiation by MCP-1 and M-CSF [1]. The concerted gene expression in endothelial activation is regulated by redox-sensitive transcription factors, in particular nuclear factor (NF)- κ B [4–6]. However, other transcription factors, such as activator protein (AP)-1, are required for the full induction of inflammatory genes [7].

Some studies have shown an active role for wine polyphenols in the reduction of atherosclerotic risk factors as well as inflammatory markers of atherosclerosis [8–12]. Wine polyphenols can reduce the susceptibility of LDL to oxidation, counteracting a key mechanism of atherosclerosis [13]. Moreover, they may exert antithrombotic effects by inhibiting platelet aggregation [14] and can improve endothelial dysfunction [15]. However, the specific red wine polyphenols, as single or blend, and the molecular mechanisms underlying the vascular anti-inflammatory effects have not been elucidated.

The phenolic composition of wines depends on the grape variety but also on other factors such as geographical location, soil, weather and winemaking conditions [16, 17]. Red wines contain relatively high levels of flavonoids and phenolic acids which are also reported as common microbiota metabolites formed by all wine polyphenols, with the exception of stilbenes [18]. Red wine polyphenols are generally present in blood and tissue as conjugated metabolites. However, inflammatory processes can activate enzymes that can release native polyphenol aglycone [19].

Therefore, in this study, we characterized the vascular anti-inflammatory properties of polyphenolic extracts from two typical South Italy (Apulia) red wines, Primitivo and Negroamaro, at physiological and non-toxic supra-physiological concentrations. We studied the effect of Primitivo and Negroamaro wine polyphenol extracts (PWPE and NWPE, respectively) on endothelial activation, by analyzing endothelium–monocyte adhesion, the endothelial expression of adhesion molecules (ICAM-1, VCAM-1 and E-Selectin), chemokine (MCP-1) and growth factor (M-CSF). We also investigated the intracellular oxidative stress and the activation of the transcriptional factors NF- κ B and AP-1. Finally, we dissected the anti-inflammatory properties of individual polyphenols present in Apulia

red wine extracts including hydroxycinnamic acids, flavonols and stilbenes, and we analyzed underlying mechanism of action in inflamed endothelium.

Materials and methods

Reagents

Trans-resveratrol (RSV) and *trans*-piceid (PCD) were obtained from ICN Biomedicals (South Chillicothe Road, Aurora, Ohio), myricetin (MYR) and kaempferol (KMP) from Extrasynthese (Genay, France), quercetin (QRC), caffeic acid (CFF), *p*-coumaric acid (CMR) and caftaric acid (CFT) from Sigma (St. Louis, MO, USA) as well as all other reagents when not otherwise specified.

Red wine polyphenol extraction and analysis

Wine samples (10 mL) were extracted three times with methanol 99 % (5 mL) and methyl tert-butyl ether (5 mL). Supernatants were collected in a fresh tube and evaporated at 35 °C to dryness. The residue was re-dissolved in 80 % methanol. Different compounds present in wine extracts were separated by RP-HPLC DAD (Agilent 1100 HPLC apparatus). The separation was performed on C18 column (5 UltraSphere rum spherical 80 A pore, 25 mm), with a linear gradient from 20 to 60 % acetonitrile, in 40 min (solvent A: 1 % phosphoric acid, solvent B: 100 % acetonitrile) with a flow of 1 mL/min at 25 °C. The chromatographic analysis performed with UV detectors was based on the comparison of results with retention time of external standards. The metabolite concentrations were obtained by deduction from the calibration curves and were expressed in mg/L. The total phenolic content in extracts was determined at 765 nm according to the Folin–Ciocalteu colorimetric method [20], and results were expressed as milligram gallic acid equivalents per liter (mg GAEs/L). Total phenolic content from PWPE and NWPE was 324 ± 12 and 780 ± 26 μ g/mL, respectively. NWPE and PWPE dry powders were dissolved in 70 % ethanol and used at the same concentration.

Cell culture and treatment

Human umbilical vein endothelial cells (HUVEC) were harvested, characterized and maintained as described and used up to the fifth passage from primary cultures [21]. Cells were obtained from discarded umbilical vein and treated anonymously conforming with the principles outlined in the Declaration of Helsinki. The human microvascular endothelial cell line (HMEC), obtained from Dr. Thomas J. Lawley, was cultured as described [22].

All experiments, when not otherwise specified, were performed in HUVEC, but control experiments were also done in HMEC-1, with identical results. For treatment, confluent endothelial cells were shifted to medium supplemented with 2.5 % FBS and incubated in the absence or presence of increasing concentrations of PWPE or NWPE (1, 10, 50 μg GAEs/mL) or pure polyphenols (1, 10, 25 $\mu\text{mol/L}$) for 1 h and then triggered with lipopolysaccharide (LPS) (0.5 $\mu\text{g/mL}$) for additional 1–24 h. Stock solutions of polyphenols (100 mmol/L) included RSV, CFF, CMR and CFT in absolute ethanol, PCD in 70 % ethanol, KMP, QRC and MYR in DMSO. As vehicle control, HUVEC were incubated with an appropriate amount of each solvent (<0.025 % v/v). These concentrations of ethanol or DMSO had no effect on any of the parameters measured in this study. Cellular toxicity by treatments was checked through a variety of techniques including cell count, morphology, protein content and MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assays.

Monocytoid cell adhesion assays

The human monocytic cell line U937 was purchased from the American Type Culture Collection (Rockville, MD) and grown in RPMI medium 1640 (Gibco BRL, Gaithersburg, MD) containing 10 % FBS. HUVEC were grown to confluence in 6-well tissue culture plates, incubated in the absence (vehicle) or presence of PWPE or NWPE (1, 10, 50 $\mu\text{g/mL}$) or pure polyphenols (1, 10, 25 $\mu\text{mol/L}$) for 1 h, and/or stimulated with LPS (0.5 $\mu\text{g/mL}$) for additional 16 h. U937 cells were labeled with 1 $\mu\text{mol/L}$ calcein AM (Molecular probe) for 30 min in RPMI medium 1640 (Gibco BRL, Gaithersburg, MD) containing 2.5 % FBS. In co-culture system, labeled U937 were seeded at 5×10^5 cell density onto HUVEC monolayer and incubated under rotating conditions (63 rpm) at 21 °C, as described [21]. After washing, the adherent U937 cells were observed under fluorescence microscope (Diagnostic Instruments) by obtaining five photomicrographs from each well and then counted using the NIH 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 cell surface molecules

HUVEC were grown to confluence in 96-well tissue culture plates, incubated in the absence (vehicle) or presence of PWPE and NWPE (1, 10, 50 $\mu\text{g/mL}$) or pure polyphenols (1, 10, 25 $\mu\text{mol/L}$) for 1 h, and/or stimulated with LPS (0.5 $\mu\text{g/mL}$) for additional 6 h for E-Selectin and 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 antihuman monoclonal antibodies against VCAM-1 (Millipore), 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 [21].

Release of MCP-1 and M-CSF

HUVEC were grown to confluence, incubated in the absence (vehicle) or presence of PWPE or NWPE (1, 10, 50 $\mu\text{g/mL}$) or pure polyphenols (10 $\mu\text{mol/L}$) for 1 h, and/or stimulated with LPS (0.5 $\mu\text{g/mL}$) for 24 h. The conditioned media were collected, and secreted MCP-1 and M-CSF levels were determined by human MCP-1 or M-CSF ELISA kit (Boster Biological Technology, LTD), as specified by the manufacturer. Absorbance was measured spectrophotometrically at 450 nm by a microplate reader.

RNA isolation and real-time quantitative PCR

Total RNA was extracted from 1×10^6 HUVEC using the Qiagen RNeasy kit (Qiagen, Milan, Italy) according to manufacturer's instructions. RNA was quantified spectrophotometrically and stored at -80 °C. For real-time quantitative reverse transcription polymerase chain reaction, 2 μg total RNA was converted into first-strand cDNA by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Monza, Italy). Quantitative PCR was performed in an Applied Biosystems 7500 by using SYBR Green PCR Master Mix (Applied Biosystems). The human VCAM-1, ICAM-1, E-Selectin, MCP-1 and M-CSF cDNA fragments were amplified using primers synthesized by Sigma Genosys and reported in Table 1. All reactions were done in triplicate, and the amount of mRNA was calculated by comparative critical threshold method. To account for possible variations related to cDNA input or the presence of PCR inhibitors, the endogenous reference ribosomal RNA 18 S was simultaneously quantified for each sample, and the data normalized accordingly. Results are expressed as fold increase relative to unstimulated control (=1).

Preparation of nuclear protein extracts and NF- κ B and AP-1 activation assay

HUVEC (1×10^6) were treated with PWPE or NWPE (1, 10, 50 $\mu\text{g/mL}$) or pure polyphenols (10 $\mu\text{mol/L}$) for 1 h and stimulated with LPS (0.5 $\mu\text{g/mL}$) for additional 1 h, after which nuclear proteins were purified by using a kit from Active Motif (Carlsbad, CA, USA). Briefly, cell monolayers were washed, collected in ice-cold PBS in the presence of phosphatase inhibitors and then centrifuged at 300 g for 5 min. Pellets were resuspended in a hypotonic buffer, treated with detergent and centrifuged at 14,000 g

Table 1 Primers sequences of real-time quantitative PCR

Gene	Accession number	Primers	Size (bp)
ICAM-1	NM_000201.2	5'-AGACATAGCCCCACCATGAG-3' 5'-CAAGGGTTGGGGTCAGTAGA-3'	190
VCAM-1	NM_00107B.3	5'-CATGGAATTTCGAACCCAAAC-3' 5'-CCTGGCTCAAGCATGTCATA-3'	140
E-Selectin	NM_000450.2	5'-ATGTGAAGCTGTGAGATGCG-3' 5'-CCACTGCAGCTCATGTTGAT-3'	252
MCP-1	NM_002982.3	5'-CCCCAGTCACCTGCTGTTAT-3' 5'-TCCTGAACCCACTTCTGCTT-3'	166
M-CSF	NM_000757.4	5'-TGGACGCACAGAACAGTCTC-3' 5'-CCTCCAGGGCTCACAATAAA-3'	235
18 ribosomal RNA	NR_003286.2	5'-AAACGGCTACCACATCCAAG-3' 5'-CCTCCAATGGATCCTCGTTA-3'	155

for 30 s. After the cytoplasmic fraction had been harvested, nuclei were lysed, and nuclear proteins solubilized in lysis buffer containing proteasome inhibitors, and protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories). The DNA-binding activity of NF- κ B (p65) and AP-1 (c-Jun and c-Fos) was quantitated using the Active Motif's ELISA-based "TransAM kit" following the manufacturer's instructions.

Detection of intracellular ROS production

The evaluation of the intracellular production of ROS in endothelial cells was performed as previously described [22], with slight modifications. Endothelial cell monolayers, grown in 6-well plates, were treated with PWPE and NWPE (1, 10, 50 μ g/mL) or pure polyphenols (1, 10, 25 μ mol/L) for 1 h and stimulated with LPS (0.5 μ g/mL) for 1 h in 2.5 % serum-containing phenol red-free medium. HUVEC were washed and then incubated with 5 μ mol/L carboxy-H₂DCFDA in Krebs–Ringer buffer for 20 min at 37 °C. After this step, cells were washed, collected in 1 mL Krebs–Ringer buffer and the fluorescence immediately read on a fluorometer (Fluoroskan II, Labsystem, Helsinki, Finland) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Fluorescence microscopy was used in some experiments to qualitatively assess the formation of intracellular ROS in response to treatment with polyphenols and/or LPS. HUVEC grown on microscope slide were epi-illuminated with a 100 watt Hg lamp and photographed using 490 nm excitation and 520 nm emission filters, respectively. Since illumination causes increased fluorescence emission because of the oxidation of the fluorescent dye, each field was exposed to light for exactly the same time (30 s).

Statistical analysis

Values are expressed as mean \pm SD. 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.

Results

Red wine polyphenolic extracts prevent monocyte adhesion and endothelial activation

We investigated the effects of PWPE and NWPE on monocyte adhesion to endothelial cells, an obliged step in inflammatory and atherosclerotic process. For this purpose, human endothelial cells were treated with different concentrations of PWPE and NWPE (1, 10, 50 μ g/mL), before stimulation with LPS (0.5 μ g/mL). The percentage of cell adhesion was evaluated by quantification of calcein staining method (Fig. 1a, b). PWPE and NWPE did not modify the U937 adhesion to unstimulated HUVEC. However, U937 adhesion to LPS-stimulated HUVEC increased about eightfold compared to control. Pre-treatment of endothelial cells with PWPE and NWPE decreased LPS-induced U937 adhesion in a concentration-dependent manner, with a significant reduction already at a concentration as low as 1 μ g/mL. The inhibition of monocyte adhesion to endothelial cells by PWPE and NWPE was not the result of cytotoxicity, as determined by MTT assay, protein content and cell count (data not shown). Similar results were obtained in microvascular endothelial cells HMEC (data not shown). Since endothelium–monocyte adhesion is mediated by enhanced expression of endothelial adhesion molecules, we investigated the effect of PWPE and NWPE on LPS-induced expression of VCAM-1, ICAM-1 and E-Selectin by cell surface EIA. All three endothelial adhesion molecules were expressed at low levels in unstimulated HUVEC, and their expression was strongly increased

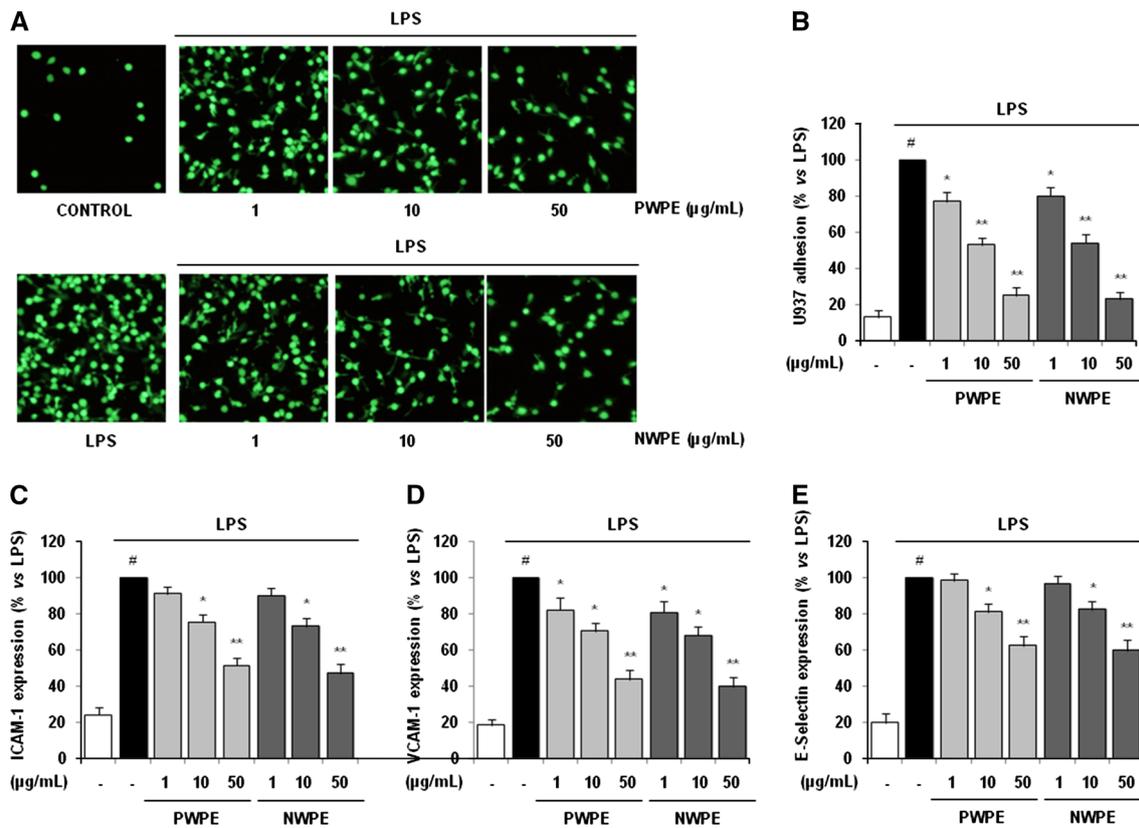


Fig. 1 Inhibitory effects of PWPE and NWPE on the monocyte adhesion to endothelial monolayer and on the expression of endothelial adhesion molecules. **a, b** HUVEC were pre-treated with Primitivo wine polyphenol extract (PWPE) and Negroamaro wine polyphenol extract (NWPE) (1–50 µg/mL) or vehicle (control) for 1 h and then stimulated with LPS 0.5 µg/mL for 16 h. HUVEC were co-cultured with calcein AM-labeled U937 monocytes for 1 h. The number of

adherent U937 cells was monitored by fluorescence microscope (**a**) or measured by the fluorescence plate reader (**b**). **c–e** Cell surface expression of ICAM-1 (**c**), VCAM-1 (**d**) and E-Selectin (**e**) was analyzed by cell surface EIA. Each experiment was performed in triplicate. Data are expressed as the percentage of LPS-induced expression (mean ± SD). #*p* < 0.01 versus control; **p* < 0.05; ***p* < 0.01 versus LPS alone

in LPS-stimulated cells. The results showed that PWPE and NWPE were able to down-regulate the endothelial cell adhesion molecule expression in a concentration-dependent manner with a significant effect already at 1 µg/mL for VCAM-1 and 10 µg/mL for ICAM-1 and E-Selectin (Fig. 1c–e), without affecting the expression of the constitutive endothelial surface antigen E1/1 (data not shown). Overall, our findings showed that PWPE and NWPE significantly prevented endothelium–monocyte adhesion by suppressing the coordinated expression of endothelial adhesion molecules VCAM-1, ICAM-1 and E-Selectin.

Structurally different red wine polyphenols inhibit the surface expression of endothelial adhesion molecules

As reported in Fig. 2a, the quantitative and qualitative analysis of PWPE and NWPE showed different distributions of polyphenols among hydroxycinnamic acids, flavonols and stilbenes. To dissect the specific contribution of structurally unrelated polyphenols present in PWPE and NWPE

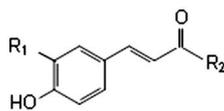
(Fig. 2b), we investigated their effects on endothelial activation. To this purpose, HUVEC were treated with chemically synthesized polyphenols (1, 10, 25 µmol/L), including hydroxycinnamic acids (CFF, CMR, CFT), flavonols (KMP, QRC, MYR) or stilbenes (RSV, PCD), before stimulation with LPS. The expression of ICAM-1, VCAM-1 and E-Selectin was evaluated by cell surface EIA. As shown in Fig. 3a–c, the hydroxycinnamic acids had different efficacy, being CMR the most effective in reducing all endothelial adhesion molecules. CFF was effective only in VCAM-1 and E-Selectin inhibition. CFT was devoid of any effect. All tested flavonols inhibited the LPS-stimulated expression of endothelial adhesion molecules in a concentration-dependent manner (Fig. 3a–c). The degree of inhibition was in the following order: KMP > QRC > MYR. Among stilbenes, RSV was able to reduce ICAM-1, VCAM-1 and E-Selectin in a concentration-dependent manner, while PCD only inhibited VCAM-1 and E-Selectin. Since 10 µmol/L was the lowest concentration effective in inhibiting endothelial activation, this dose was chosen

A

GROUPS	POLYPHENOLS	PWPE		NWPE	
		µg/mL	µmol/L	µg/mL	µmol/L
HYDROXYCINNAMIC ACIDS	<i>p</i> -Coumaric acid	0.04 ± 0.03	0.24	0.04 ± 0.02	0.27
	Caffeic acid	1.26 ± 0.54	6.98	1.52 ± 0.81	8.41
	Caftaric acid	8.23 ± 1.12	26.37	8.01 ± 0.98	25.64
Total Hydroxycinnamic Acids		9.53		9.57	
FLAVONOLS	Kaempferol	0.05 ± 0.02	0.18	0.08 ± 0.04	0.30
	Quercetin	0.13 ± 0.05	0.42	0.14 ± 0.03	0.47
	Myricetin	0.04 ± 0.01	0.12	0.09 ± 0.02	0.29
Total Flavonols		0.22		0.31	
STILBENES	<i>trans</i> -Resveratrol	0.06 ± 0.02	0.24	0.02 ± 0.01	0.10
	<i>trans</i> -Piceid	0.20 ± 0.05	0.51	0.09 ± 0.03	0.24
Total Stilbenes		0.26		0.11	
TOTAL POLYPHENOLS		10.00		10.00	

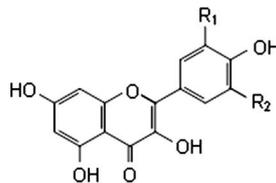
B

HYDROXYCINNAMIC ACIDS



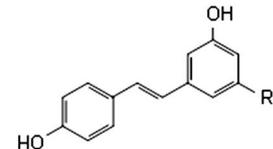
	R ₁	R ₂
<i>p</i> -Coumaric acid (CMR)	H	OH
Caffeic acid (CFF)	OH	OH
Caftaric acid (CFT)	OH	C ₄ O ₆ H ₅

FLAVONOLS



	R ₁	R ₂
Kaempferol (KMP)	H	H
Quercetin (QRC)	OH	H
Myricetin (MYR)	OH	OH

STILBENES



	R ₁
<i>trans</i> -Resveratrol (RSV)	OH
<i>trans</i> -Piceid (PCD)	OGlucose

Fig. 2 Characterization of PWPE and NWPE polyphenol content and chemical structure of polyphenols. **a** Polyphenol content PWPE and NWPE (10 µg/mL). **b** Chemical structures of polyphenol groups identified in red wine extracts: hydroxycinnamic acids (caffeic acid,

CFF; *p*-coumaric acid, CMR; caftaric acid, CFT), flavonols (kaempferol, KMP; quercetin, QRC; myricetin, MYR) and stilbenes (*trans*-resveratrol, RSV; *trans*-piceid, PCD)

for subsequent assays as well as for the characterization of the molecular mechanism of actions.

Red wine polyphenols reduce the expression of endothelial adhesion molecules at a transcriptional level

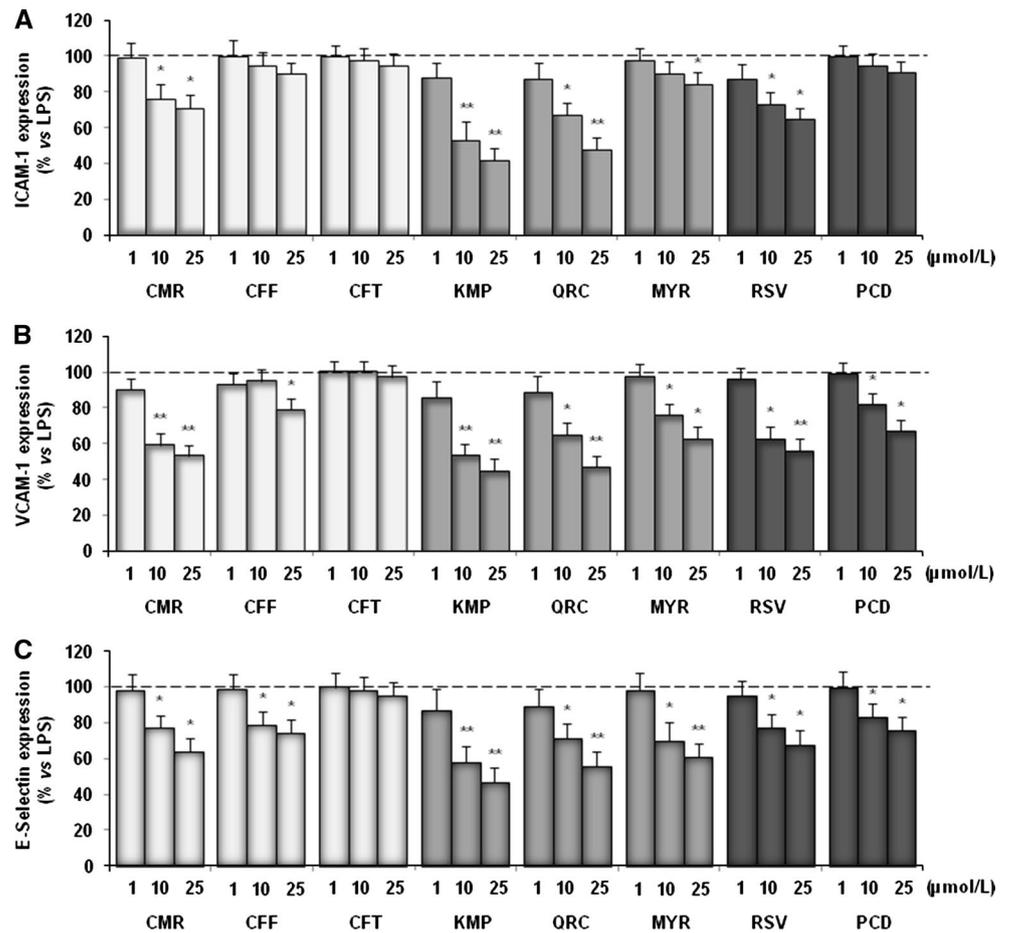
To verify whether the inhibition of cell surface expression of endothelial adhesion molecules was consequent to decreased mRNA levels, we investigated the effects of PWPE, NWPE or pure polyphenols on ICAM-1, VCAM-1 and E-Selectin mRNA levels. For this purpose, HUVEC were incubated with PWPE, NWPE (1, 10, 50 µg/mL) or pure polyphenols (10 µmol/L) for 1 h before stimulation with LPS for 4 h. Cell adhesion molecules mRNA levels were measured by real-time quantitative RT-PCR (Fig. 4a–f). Consistent with reduced protein levels of adhesion molecules, pre-treatment of endothelial cells with PWPE and NWPE also decreased

ICAM-1, VCAM-1 and E-Selectin mRNA levels (Fig. 4a–c). Accordingly, the hydroxycinnamic acids CMR and CFF, all tested flavonols and, among stilbenes, RSV also inhibited mRNA levels of ICAM-1, VCAM-1 and E-Selectin (Fig. 4d–f). PCD inhibited only VCAM-1 and E-Selectin and CFT did not exhibit significant effects.

Red wine polyphenols decrease MCP-1 and M-CSF expression

Since endothelial activation is accompanied by the release of chemokines and growth factors, we investigated the potential effects of red wine polyphenols on the expression of MCP-1 and M-CSF at both the protein and mRNA levels. To this purpose, HUVEC were treated for 1 h with PWPE, NWPE or pure polyphenols before stimulation with LPS, after which MCP-1 and M-CSF mRNA or secreted

Fig. 3 Differential effects of pure polyphenols on the stimulated expression of the endothelial adhesion molecules ICAM-1, VCAM-1 and E-Selectin. HUVEC were pre-treated with increasing concentrations of each polyphenol (1–25 $\mu\text{mol/L}$) for 1 h and then stimulated with LPS for 16 h for ICAM-1 and VCAM-1 or 6 h for E-Selectin. Cell surface expression of ICAM-1 (a), VCAM-1 (b) and E-Selectin (c) was analyzed by cell surface EIA. Each experiment was performed in triplicate. Data are expressed as percentage of LPS-induced expression (mean \pm SD). LPS expression is specified by dashed line. * $p < 0.05$; ** $p < 0.01$ versus LPS alone



proteins were analyzed. Unstimulated HUVEC expressed and released MCP-1 and M-CSF at low levels. LPS stimulation induced MCP-1 and M-CSF protein release and mRNA levels (Fig. 5a, b). PWPE and NWPE reduced MCP-1 and M-CSF both at protein and mRNA levels in a concentration-dependent manner (Fig. 5a, b). The effects of pure polyphenols on MCP-1 and M-CSF protein and mRNA levels are shown in Fig. 5c, d. MCP-1 protein release was significantly inhibited by CMR, flavonols and stilbenes, while M-CSF protein release was reduced only by flavonols and RSV (Fig. 5c). The same results were observed for MCP-1 and M-CSF mRNA levels (Fig. 5d). CFF and CFT were devoid of any effect on either MCP-1 or M-CSF (Fig. 5c, d).

Red wine polyphenols prevent LPS-induced NF- κ B and AP-1 activation

Since the expression of pro-inflammatory genes including VCAM-1, ICAM-1, E-Selectin, MCP-1 and M-CSF is regulated by redox-sensitive transcription factors such as NF- κ B and AP-1 [6, 7], we investigated the effects of PWPE, NWPE or pure polyphenols on LPS-stimulated

NF- κ B and AP-1 activation by an ELISA-based method. We evaluated NF- κ B activation by measuring the DNA-binding activity of the NF- κ B subunit p65 and AP-1 activation by measuring the DNA-binding activity of the AP-1 subunits c-Fos and c-Jun. NF- κ B and AP-1 activation was low in unstimulated HUVEC, but increased by about threefold and twofold, respectively, in LPS-stimulated HUVEC (Fig. 6a, b). PWPE, NWPE and chemically synthesized polyphenols, except CFT, significantly inhibited the activation of NF- κ B p65 and, to a lesser extent, the activation of AP-1 c-Fos and c-Jun (Fig. 6a–d). These results suggest that red wine polyphenol extracts and unrelated pure polyphenols differentially modulate the activation of NF- κ B and AP-1 in response to inflammatory stimuli in endothelial cells.

Red wine polyphenols reduce intracellular ROS levels in activated endothelium

Changes in the cellular redox balance may alter the activation of NF- κ B and AP-1, which can be inhibited by treatment with antioxidants [4]. Since PWPE and NWPE exhibited strong antioxidant potential in cell-free systems (data

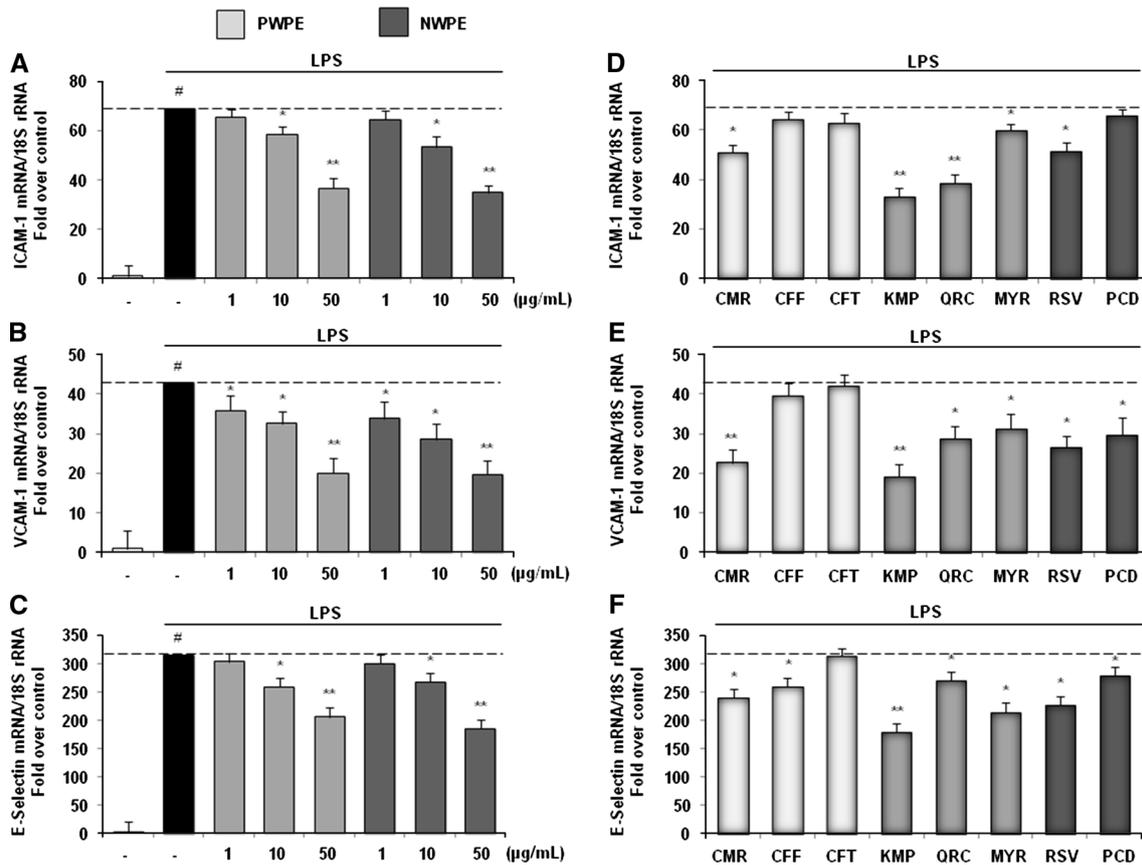


Fig. 4 Inhibitory effects of PWPE, NWPE and pure polyphenols on ICAM-1, VCAM-1 and E-Selectin mRNA levels. HUVEC were pre-treated with PWPE and NWPE (1–50 $\mu\text{g}/\text{mL}$) (a–c) or pure polyphenols (10 $\mu\text{mol}/\text{L}$) (e, f) for 1 h and then stimulated with LPS for 4 h. ICAM-1, VCAM-1 and E-Selectin mRNA levels were determined

by quantitative RT-PCR. Data are representative of three independent experiments and expressed as fold over unstimulated control (mean \pm SD). LPS expression is specified by *dashed line*. # $p < 0.01$ versus control; * $p < 0.05$; ** $p < 0.01$ versus LPS alone

not shown) as well as pure polyphenols [23], we verified their antioxidant capability in endothelial cells triggered with LPS. As shown in Fig. 7a, c, LPS yielded a great number of ROS in HUVEC, as assessed by using the ROS-sensitive probe carboxy- H_2DCFDA . PWPE and NWPE and pure polyphenols significantly reduced LPS-stimulated intracellular ROS levels (Fig. 7a–d). In contrast to the inhibition of markers of endothelial activation, all polyphenols exhibited a similar strong antioxidant activity.

Discussion

In the present study, we dissect the anti-inflammatory properties of polyphenolic extracts from two red wines typical of South Italy, Primitivo and Negroamaro. We demonstrate that PWPE and NWPE inhibited monocyte adhesion to LPS-stimulated endothelial cells by down-regulating the expression of adhesion molecules, ICAM-1, VCAM-1, E-Selectin, as well as chemoattractants, MCP-1 and

M-CSF, both at mRNA and protein levels. Our findings are in agreement with studies in humans and animal models showing that polyphenols of red wine reduced leukocyte adhesion molecules and soluble inflammatory mediators related to atherosclerosis [9, 24, 25]. However, we here provided new evidence of the anti-atherogenic effects of red wine polyphenols having shown an inhibitory effect also on the expression of growth factors such as M-CSF. M-CSF is a cytokine chemotactic for monocytes and is expressed at high levels in atherosclerotic lesions. Both endothelial cells and macrophages can produce M-CSF as induced by oxidized LDL and pro-inflammatory molecules such as bacterial LPS [26]. Bone marrow transplantations experiments suggested that artery wall-derived M-CSF is the primary source of circulating M-CSF and is the key source of M-CSF as it relates to atherosclerosis [27]. M-CSF in addition to MCP-1 is responsible for monocyte migration into the artery wall, and M-CSF itself can induce endothelial cell-derived MCP-1 [28]. Our results show that polyphenolic extracts from red wines are able to inhibit the

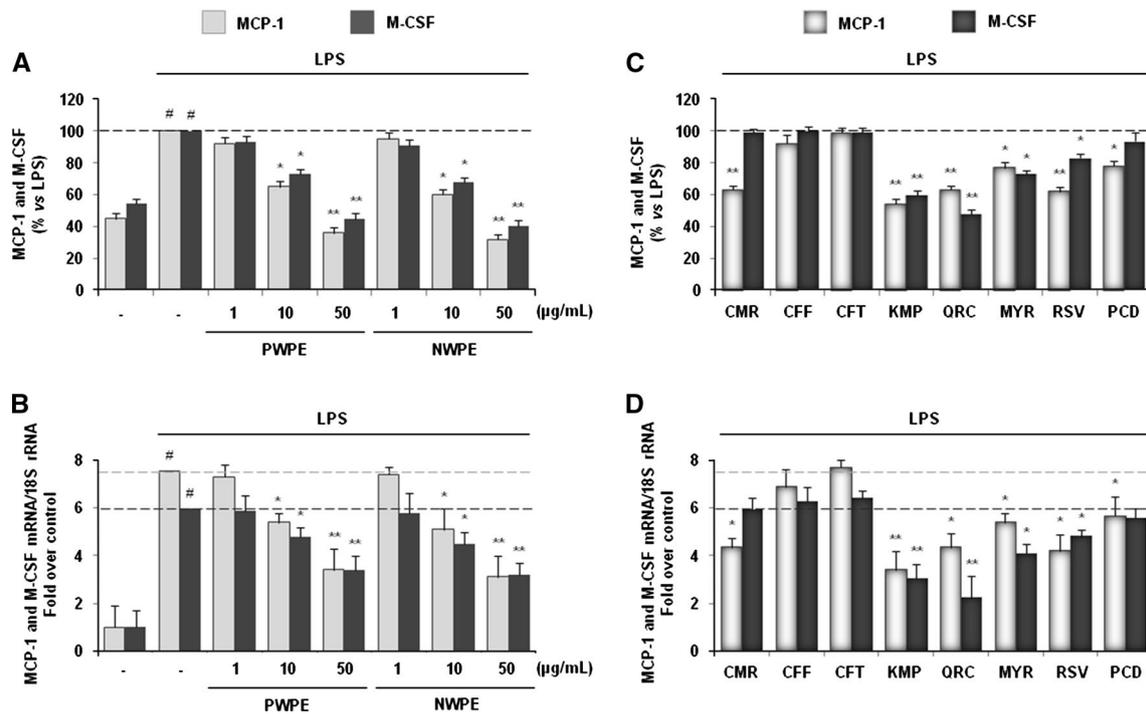


Fig. 5 Inhibitory effects of PWPE, NWPE and pure polyphenols on LPS-induced MCP-1 and M-CSF protein and mRNA levels. HUVEC were pre-treated with PWPE and NWPE (1–50 µg/mL) (a, b) or pure polyphenols (10 µmol/L) (c, d) for 1 h and then stimulated with LPS for 4 h or 24 h. a, c MCP-1 and M-CSF protein release was determined in culture medium by ELISA. Results are reported as percentage of LPS-induced expression (mean ± SD) as specified by dashed

line. b, d MCP-1 and M-CSF mRNA levels were evaluated by quantitative RT-PCR and are expressed as fold over unstimulated control (mean ± SD). LPS-induced MCP-1 levels are indicated as light gray dashed line. LPS-induced M-CSF levels are indicated as dark gray dashed line. Data are representative of three independent experiments. #*p* < 0.01 versus control; **p* < 0.05; ***p* < 0.01 versus LPS alone

expression and the release of M-CSF by endothelial cells in addition to reduce the expression of adhesion molecules and chemoattractants. These findings highlight a multiple anti-atherosclerotic role of polyphenols, which are able to counteract the adhesion of monocytes and potentially their maturation and differentiation.

Interestingly, our results complement other reports on the immunomodulating properties of red wine polyphenols [29]. Recent in vitro studies [30] showed the ability of wine polyphenolic extracts to polarize the immune response toward an anti-inflammatory pathway, to induce T regulatory cells, which can contribute to modulate atherosclerosis development and progression, and to mitigate chronic inflammatory diseases.

Red wine polyphenolic extracts are a complex blend of structurally different compounds, but the specific anti-inflammatory role of individual polyphenols, flavonoid and non-flavonoid, is not completely clear. We investigated the effects of polyphenols mainly represented in the PWPE and NWPE, such as flavonoids, namely flavonols (KMP, QRC, MYR) as well as non-flavonoids such as hydroxycinnamic acids (CMR, CFF, CFT) and stilbenes (RSV, PCD), on the endothelial expression of adhesion molecules,

chemoattractants and growth factors. We found that both flavonols and stilbenes significantly reduced the endothelial expression and production of M-CSF, while the hydroxycinnamic acids were devoid of any significant effect in our experimental conditions. The more effective polyphenols were QRC and KMP followed by MYR and RSV. To our knowledge, the present data are the first demonstration of the differential effect of red wine polyphenols on endothelial M-CSF release and expression. Indeed, there are only few studies showing M-CSF reduction by RSV in human macrophages [31] and in lung epithelial cells [32], but data on endothelial cells are lacking. In addition, we found that all polyphenol groups inhibited the endothelial expression of adhesion molecules and MCP-1, although with different potency. Flavonols were more effectual than stilbenes, which were more effective than hydroxycinnamic acids. Among the flavonols, KMP was more potent than QRC, followed by MYR. These differential activities by flavonols can be explained by their peculiar structures. As KMP has only one –OH moiety in the B-ring, while QRC and MYR have two and three –OH moieties, respectively; the stronger inhibitory effect by KMP may be related to a minor number of –OH moieties and a major lipophilicity and a higher

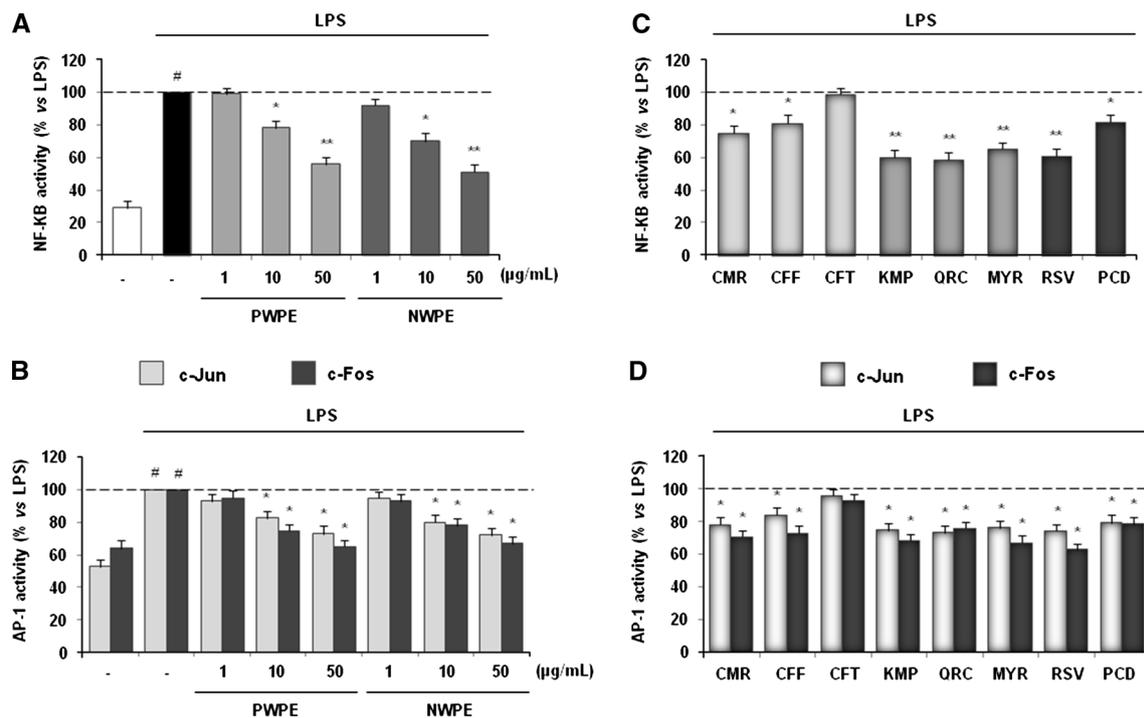


Fig. 6 Effects of PWPE, NWPE and pure polyphenols on LPS-induced NF- κ B and AP-1 activation. HUVEC were pre-treated with PWPE and NWPE (1–50 μ g/mL) (a, b) or pure polyphenols (10 μ mol/L) (c, d) for 1 h and then stimulated with LPS for further 1 h, after which nuclear proteins were assayed by an ELISA-based

method measuring the DNA-binding activity of NF- κ B p65 (a, c) and AP-1 c-Fos and c-Jun (b, d). Each experiment was performed in triplicate. Data are expressed as the percentage of LPS-induced expression (mean \pm SD) as specified by *dashed line*. #*p* < 0.01 versus control; **p* < 0.05; ***p* < 0.01 versus LPS alone

permeability through the plasma membrane [33, 34]. Concerning stilbenes, RSV exerted a higher inhibitory effect than PCD [35]. This finding could be partially explained by a different cellular uptake: RSV can pass through cell membrane, whereas PCD transport is likely active and involves the transporter sodium-dependent glucose cotransporter-1, whose role has been recently confirmed also in endothelial cells [36]. Among the hydroxycinnamic acids, CFT was devoid of any inhibitory effect on all endothelial inflammatory markers, while CMR was more effective than CFF in the inhibition of endothelial adhesion molecules and MCP-1 expression. The major effectiveness of CMR could be related to its major cellular uptake and higher affinity for monocarboxylic acid transporter in comparison to CFF [37]. Our findings show a novel inhibitory effect on MCP-1 synthesis and secretion in endothelial cells by CMR, KMP and MYR, and they are consistent with previous data on RSV [38], PCD [35], QRC [39] and CFF [40], contributing to explain the anti-inflammatory action of polyphenols in the context of the Mediterranean diet.

It is well known that the expression of pro-inflammatory genes including VCAM-1, ICAM-1, E-Selectin as well as MCP-1 and M-CSF is regulated by the activation of the redox-sensitive transcription factors NF- κ B and AP-1. Indeed, binding site for NF- κ B and AP-1 is present in the

promoter of these genes and is involved in their up-regulation [4, 6, 41]. We showed that PWPE and NWPE inhibited the activation of NF- κ B and, to a lesser extent, AP-1. All pure polyphenols, except CFT, also reduced NF- κ B and AP-1 activation. Flavonols and RSV showed a greater inhibition of NF- κ B activation compared with other polyphenols. These results confirmed and extended previous *in vitro* [22, 33, 42, 43] and *in vivo* [24, 44] data on NF- κ B and AP-1 regulation by red wine polyphenols as a mechanism for the modulation of inflammation and immune response in atherosclerosis and chronic inflammatory diseases. Since oxidative stress can regulate inflammatory gene expression in the vasculature during atherogenesis [45], we investigated the intracellular antioxidant potential of PWPE, NWPE and pure polyphenols in inflamed conditions. In accordance with Lotito [46], we did not find any relationship between the intracellular antioxidant capacity of polyphenols and their effects on inflammatory markers in endothelial cells. The endothelial oxidative stress was significantly decreased by all pure polyphenols at a similar level, in contrast to their different antioxidant potential in cell-free systems [23]. These findings suggested that red wine polyphenols could act at multiple levels either as direct antioxidants or as modulators of inflammatory gene expression. This last mechanism could be in part explained

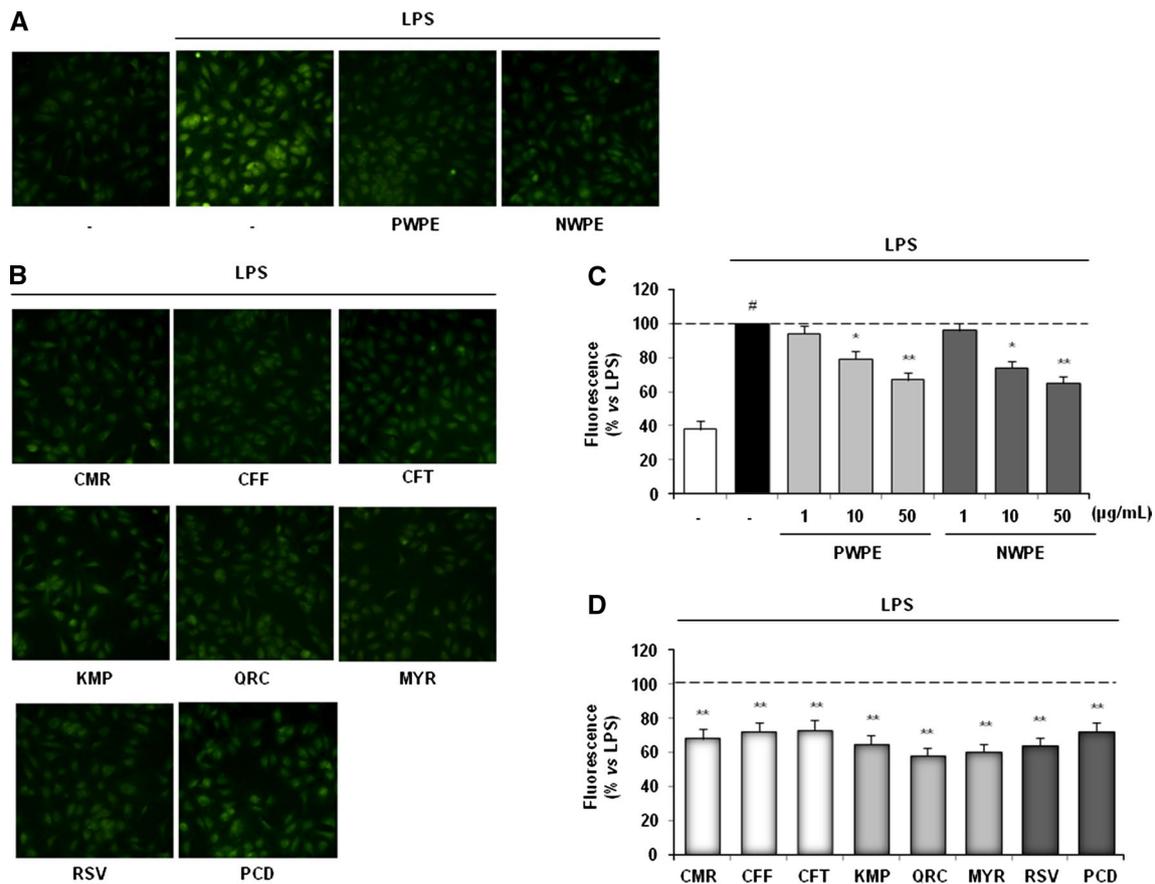


Fig. 7 Effects of PWPE, NWPE and pure polyphenols on LPS-induced intracellular levels of ROS. HUVEC were pre-treated with PWPE and NWPE (1–50 µg/mL) or pure polyphenols (10 µmol/L) for 1 h and then stimulated with LPS for further 1 h, after which intracellular levels of ROS were assayed by using carboxy-H₂DCFDA staining by fluorescence microscope (a, b) and by fluo-

rescence plate reader (c, d). In A, the effect of 10 µg/mL of PWPE and NWPE is shown. Each experiment was performed in triplicate. Data are expressed as the percentage of LPS-induced expression (mean ± SD) as specified by dashed line. #*p* < 0.01 versus control; **p* < 0.05; ***p* < 0.01 versus LPS alone

by the effects of red wine polyphenols on the reduced activation of redox-sensitive transcription factors such as NF-κB and AP-1 [47, 48].

As a possible relevance of our findings, we found that the lowest effective concentration of polyphenolic extracts is in the same order of magnitude that could be attained in vivo [49]. Furthermore, the inhibitory effects of pure polyphenols occurred at concentrations higher than those actually present in the extracts (Fig. 2a), suggesting the occurrence of a synergism among different polyphenols. Interestingly, recent studies have shown that purified polyphenols, distributed as dietary supplements, do not have the same effects in promoting health as the same phytonutrients in the food context [50]. The nutritional context could influence the effects of polyphenols by affecting their bioavailability or supporting synergistic interactions on multiple targets [51].

However, a limitation of the present study is that our findings derived from in vitro models; therefore, they could not be directly extrapolated in humans. However,

the study has been performed by using human cultured endothelial cells (HUVEC and HMEC), which represent cellular models pathophysiology relevant in inflammation and atherosclerosis. Moreover, endothelial cell cultures allow more controlled conditions of specific biochemical events and are useful for detailed mechanistic studies. Even though the forms of polyphenols appearing in the circulation after absorption could be different from those found in red wine and used in this study, free polyphenols (i.e., their aglycones) can be locally generated in vivo from their metabolites at sites of inflammation, for example, by the action of glucuronidases and sulfatases [19]. Noteworthy, the biological effects of hydroxycinnamic acids could be relevant in vivo because they can be obtained not only through a direct consumption but also by a gut microflora metabolism [18, 52].

In conclusion, this in vitro study showed new anti-inflammatory and anti-atherosclerotic properties of red wine polyphenolic extracts and identified specific

bioactive compounds which could provide novel preventive or therapeutic opportunities to counteract inflammatory diseases including atherosclerosis.

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

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