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Chemical composition and "*in vitro*" anti-inflammatory activity of *Vitis vinifera* L. (var. Sangiovese) tendrils extract

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A B S T R A C T

Grapevine (*Vitis vinifera* L.) is currently used for wine production, herbal medicine and food supplements. In this study we investigated the phenolic composition of grape tendril extract (TVV), because these compounds are probably responsible for the observed biological effects of TVV. We performed our investigation on cells implicated in the inflammatory process and in vascular damage, such as monocyte (U937) and senescent endothelial (HUVEC) cells. We analysed changes in microRNA (miRNA) expression during a pro-inflammatory condition in LPS (lipopolysaccharide) stimulated cells. MiR-126, defined as angiomiR for its ability in the maintenance of endothelial cell functions, and miR-146a, defined as inflamma-miR, for its ability to down-modulate (NF-kB nuclear factor-kB) pro-inflammatory pathway, were analysed along with their targets. TVV extract (40 μg/mL) was able to prevent the LPS stimulus and may play an important role in the prevention of endothelial dysfunction when the accumulation of senescent endothelial cells occurs.

Keywords:
Vitis vinifera tendrils
Anti-inflammatory activity
miR-126
miR-146a
MIRAKIL

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Abbreviations: CAD, coronary artery disease; CVD, cardiovascular disease; DPPH, diphenylpicrylhydrazyl; HPLC-DAD, high-performance liquid chromatography with diode-array detection; IRAK-1, IL-1 receptor-associated kinase 1; LPS, lipopolysaccharide; MIRAKIL, biomarkers associated to SASP (miR-146a expression, IRAK-1, IL-6 released into the culture medium); NCTC 2544, human keratinocytes; P13, endothelial cells cultured until the 13th passage; PAI-1, plasminogen activator inhibitor type 1; PBMCs, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; SASP, senescence-associated secretory phenotype; T2DM, type 2 diabetes mellitus; TRAF6, TNF receptor-associated factor 6; TVV, grape tendril extract

1. Introduction

The grapevine *Vitis vinifera* L. is a woody perennial vine plant that uses tendrils to climb. During plant development, tendrils arise from lateral meristems (growth region with proliferating cells) before the plant initiates flowering. From the same lateral meristems, inflorescence may be formed instead of tendrils if a flowering stimulation occurs. In this way, the grapevine lateral meristem can develop either as a tendril (climbing) or inflorescence (reproductive) organ (Calonje, Cubas, Martìnez-Zapater, & Carmona, 2007). *V. vinifera* L. fruit is used mostly for dietary purpose and wine production, whereas seeds and leaves of this species find applications in herbal medicine and food supplements. Seeds, grape skin and leaves have been reported to be rich in active polyphenols, such as catechins, epicatechins, procyanidins, proanthocyanidins, resveratrol and other stilbene derivatives (Fraternale et al., 2011). Usually, polyphenols in food are present conjugated with sugars or organic acids. Many clinical, animal and in vitro studies describe the beneficial effects of these phytochemicals. High intake of food rich in polyphenols has been associated with a decreased variety of human chronic disorders: cardiovascular disease, inflammatory and metabolic diseases, and neurodegenerative diseases (Milenkovic, Jude, & Morand, 2013). Polyphenolic compounds from red muscadine grape (*Vitis rotundifolia* Michx.) have been demonstrated to have protective effects on HUVEC endothelial cells against oxidative stress and inflammation (Noratto, Angel-Morales, Talcott, & Mertens-Talcott, 2011). Low concentrations of grape seed proanthocyanidins inhibited polyunsaturated fatty acid peroxidation, and this protection against free radicals was higher than that stated for vitamins C and E (Bouhamidi, Prévost, & Nouvelot, 1998). A recent study has also shown the anti-apoptotic activity of grape seed proanthocyanidin extract (Ulusoy et al., 2012). These diverse biological activities of polyphenols suggest differences in mechanism of action, which can be specific and/or non-specific. The nonspecific mechanisms are related to their interaction with plasma membrane, whereas the specific mechanisms involve their interaction with cell receptors, modulation of enzyme activities and transcription factors (Milenkovic et al., 2013). Molecular targets common to a variety of polyphenols have been described to be effective in modulating gene expression, among them, microRNAs (miRNAs, miRs), which represent a category of gene regulators (Milenkovic et al., 2013). Recently, miRNAs have been investigated as modulators of the immune response. They are small non-coding RNAs able to suppress gene expression at a posttranscriptional level by inhibiting protein synthesis or by endorsing the degradation of their target mRNAs. MicroRNAs have already been described to be involved in the inflammatory processes (Olivieri, Lazzarini, Babini, et al., 2013). The inflammatory responses can be initiated by lipopolysaccharide (LPS) from Gram-negative bacteria activating the TLR-4 cell signalling pathway. IL-1 receptor-associated kinase 1 (IRAK-1) and TNF receptor-associated factor 6 (TRAF6) are adapter molecules in the TLR-4 signalling cascade provoking the activation of NF-kB (NF-kB nuclear factor-kB), which in turn is responsible for pro-inflammatory cytokine production. TLR-4 is able to modulate cytokine expression through a negative feedback regulation loop that involves the down-regulation of IRAK-1 and TRAF6 protein levels (Olivieri, Lazzarini, Babini, et al., 2013). MiR-126 (angiomiR, able to maintain endothelial function) and miR-146a (inflamma-miR, able to down-modulate NF-kB pro-inflammatory pathway) play an important role in the inflammation process since they are responsible for regulating cell adhesion molecules (ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion protein 1) and IRAK-1/TRAF6 expression, respectively. Red wine polyphenols (from *Vitis aestivalis* hybrid) have been described to decrease the mRNA expression of some LPS-induced inflammatory markers (NF-kB; ICAM-1, VCAM-1 and PECAM) in human colon-derived CCD-18Co myofibroblast cells. The anti-inflammatory activity has been in part mediated by the induction of miR-126, which was responsible for decreasing the expression of its VCAM-1 target (Angel-Morales, Noratto, & Mertens-Talcott, 2012a). Furthermore, in the same cellular system, other researchers have demonstrated the anti-inflammatory effect of a shrub flavonol-rich fraction by the up-regulation of miR-146a (Noratto, Kim, Talcott, & Mertens-Talcott, 2011). Polyphenolic compounds from red muscadine grape (*V. rotundifolia*) exert their anti-inflammatory properties by increasing miR-126 and decreasing VCAM-1 expressions (Noratto, Angel-Morales, et al., 2011). The same authors found similar anti-inflammatory properties in a curcuminoid extract, on HUVEC cells, where a down-regulation of NF-kB was associated with reduced miR-146a expression (Angel-Morales, Noratto, & Mertens-Talcott, 2012b). According to recent studies, inflammation and oxidative stress modulate the endothelial progenitor cell (EPC) bioactivity, leading to the production of multiprotein inflammatory complexes called inflammasomes (Salminen, Ojala, Kaarniranta, & Kauppinen, 2012). Oxidative stress and inflammation increase with ageing and are related to age-related degenerative disease, such as cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), Alzheimer disease and cancer (Harman, 2006; Salminen et al., 2012). Therefore, the detection of new nutraceuticals and phytochemical compounds with anti-oxidant and anti-inflammatory properties is of great interest. *In vivo* studies indicate that a long-term (1 year) supplementation with a grape extract (containing resveratrol extracted from Napoleon cultivar irradiated grapes) can down-regulate the expression of pro-inflammatory cytokines via miRNAs-mediated mechanism. This study has been performed in peripheral blood mononuclear cells (PBMCs) of T2DM patients, where a beneficial immunomodulatory effect has been observed (Tome-Carneiro, Larrosa, et al., 2013). The same authors, using the same resveratrol-containing grape nutraceutical, found cardiovascular benefits in patients with coronary artery disease (CAD) (Tome-Carneiro, Gonzalvez, et al., 2013). To the best of our knowledge, similar anti-inflammatory studies involving mass production and dietary supplementation of tendrils from *V. vinifera* are lacking. Only Fraternale et al. (2011) have investigated the effects of the aqueous extract of *V. vinifera* tendrils on cultured human keratinocytes (NCTC 2544) and have shown its anti-oxidant capacity; they (Fraternale et al., 2015) have also analysed the qualitative phenolic composition of a hydroalcoholic tendrils extract. For this reason, we aimed to study the chemical composition, anti-oxidant and anti-inflammatory properties of the grape tendril ethanol extract (TVV). We performed our investigation on cells implicated in the inflammation process and in vascular damage, such as monocyte (U937) and senescent endothelial (HUVEC) cells. The

analyses of miR-126, miR-146a and their related targets allowed us to evaluate the inflammatory profile of LPS-stimulated cells treated with the TVV extract.

2. Materials and methods

2.1. Plant material

The young and herbaceous fresh tendrils used in this study were collected in June 2013 from *V. vinifera* L. var. Sangiovese plants located near Urbino (Marche region, central Italy, at 381 m on sea level, GPS coordinates: latitude 43.713425, longitude 12.626819), and identified by D. Fraternale. A voucher specimen is deposited in the herbarium of the Botanical Garden of the University of Urbino "Carlo Bo" (TVV 02). Tendrils were dried in a hot air dryer at 30 °C for 72 hours and were ground through a blade mill (Moulinex AD56 42) until a fine powder that passes through a 1 mm sieve was obtained. The tendrils powder was extracted with 70% ethanol in a closed circuit during 20 h in Soxhlet (Dani et al., 2010). Subsequently, the solvent present in the extract was evaporated at 40 °C by a rotary evaporator. The extract was preserved in vials and stored at 4 °C until use.

2.2. Phenolic compounds analysis by HPLC-DAD (high-performance liquid chromatography with diode-array detection)

An Agilent 1200 chromatographic system equipped with a quaternary pump, an UV-vis diode-array detector, an automatic injector, and ChemStation software (Palo Alto, CA, USA) was used for the HPLC separation, identification and quantification of phenolic compounds. Prior to direct injection, the samples were filtered through a 0.45 μm Nylon filter (E0034, Análisis Vínicos, Spain). All analyses were made in triplicate. The separation of the phenolic compounds (low molecular weight) was carried out following the method described in Gordillo et al. (2014). Individual phenolic compounds were separated using a Zorbax C18 column (250 × 4.6 mm, 5 μm particle size) maintained at 40 °C. Acetonitrile–formic acid–water (3:10:87, v/v/v) as solvent A and acetonitrile–formic acid–water (50:10:40, v/v/v) as solvent B were used. The elution profile was as follows: 0–5 min 94% A; 5–10 min 89% A; 10–15 min 80% A; 15–20 min 77% A; 20–25 min 74% A; 25–30 min 60% A; 30–35 min 50% A; 35–38 min 40% A; 38–46 min 94% A. The flow rate was 0.63 mL/min, and the injection volume was 50 μL. UV-Vis spectra were recorded from 200 to 800 nm with a bandwidth of 2.0 nm. The wavelengths of detection were 280 nm (flavanols and benzoic acids), 320 nm (hydroxycinnamic acids and their tartaric esters), and 370 nm (flavonols). Phenolic compounds were identified by their retention time, UV-vis spectra and mass spectra data in a Finnigan LXQ Linear Ion Trap (Thermo Scientific, San Jose, CA, USA). The identification of phenolic compounds was achieved by comparison of the retention times and mass spectra with those of the available pure standards and our data library (Fraternale et al., 2015). The external calibration method was used for quantification by comparing the areas with standards of gallic, caffeic, ellagic, catechin, and quercetin. Hydroxycinnamic acids were quantified using the calibration curves of caffeic acid. Flavanols were quantified with the calibration curve of catechin while flavonols and their derivatives as quercetin. Phenolic compounds concentration was expressed as mg/1 g DW. The sum of benzoic acids, hydroxycinnamic acids, monomeric flavan-3-ols, and flavonols was also estimated by summing the content of each member identified by HPLC respectively.

2.3. Cell cultures and treatments

To evaluate the anti-oxidant properties of TVV, human monocytic U937 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (Euroclone, Milano, Italy), penicillin (50 units/mL) and streptomycin (50 μg/mL) (Euroclone) at 37 °C in T-75 tissue culture flasks (Corning, Corning, NY) gassed with an atmosphere of 95% air to 5% CO_2. Treatments were performed in 1 mL of medium containing 500×10^3 cells by adding 1 mM H_2O_2 for 1 h. The cells were then washed and resuspended in fresh complete medium, and incubated for recovery as previously described (Colussi et al., 2000). Increasing concentrations of TVV (0–1 mg/mL) were added for 5 h during the recovery. The solutions were prepared based on dilutions composed of TVV and RPMI medium. Viability of U937 and HUVEC cells was evaluated after the treatments with TVV. Cell viability was measured by trypan blue exclusion. This allowed us to calculate the percentage of viable cells in treated and untreated TVV cells. The apoptotic activity was characterised by Hoechst 33342 (Sigma-Aldrich) staining and validated by DNA fragmentation (1×10^6 cells were lysed and the purified DNA was loaded on a 1.5% agarose gel to evaluate the ladder-like apoptotic pattern). Nuclear fragmentation in several smaller fragments was detected by fluorescence microscopy on slides of Hoechst 33342 (Sigma-Aldrich) stained cell samples. The fraction of cells with fragmented nuclei among the total cell population was calculated in Hoechst 33342 stained cells, counting at least 200 cells in at least 6 random selected fields (Colussi et al., 2000). To evaluate the anti-inflammatory properties of TVV, U937 cells were differentiated into macrophages with 100 nM Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) treatment for 48 h. The cells were then incubated for 24 h without PMA. To stimulate U937 cells, 1 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich) was added to the medium for 6 h. TVV (40 μg/mL) was added 1 h before LPS stimulation. HUVECs were purchased from Clonetics and cultured in endothelial growth medium (EGM-2; both from Lonza, Basel, Switzerland). Replicative senescence was studied in endothelial cells cultured until the 13th passage (P13) as described previously (Olivieri, Lazzarini, Recchioni, et al., 2013). HUVECs were cultured in flasks at a concentration of 80,000 cells/flask (70% confluence) in EGM-2 and stimulated with 1 μg/mL LPS added to the medium for 6 h. TVV (40 μg/mL) was added 1 h before LPS stimulation.

2.4. DPPH radical assay

The anti-oxidant activity was measured by a modification of the DPPH (diphenylpicrylhydrazyl) radical-scavenging method (Cuendet, Hostettmann, Potterat, & Dyatmiko, 1997). An aliquot of the reaction solution was mixed with 80 μL of 0.5 mM DPPH (Sigma-Aldrich) in ethanol and 40 μL of the TVV dissolved in ethanol at final concentrations ranging from 0.10 to 0.50 mg/mL.

The final volume was adjusted to 400 µL with ethanol. Mixtures were vigorously shaken and left for 1 h in the dark. Absorbance was measured at 517 nm using ethanol as blank. Eighty microlitres of 0.5 mM DPPH diluted in 400 µL of ethanol was used as control. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The following equation (1) was used to calculate quenching of DPPH radical:

$$I(\%) = 100 \times (A0 - As)/A0 \quad (1)$$

where A0 is the absorbance of the control and As is the absorbance of the tested sample. The EC_{50} value represented the concentration of the sample that caused 50% quenching. All tests and analyses were run in triplicate and mean values were recorded. L-ascorbic acid (Sigma-Aldrich) served as positive control at a concentration of 0.040 mg/mL.

2.5. *Senescence-associated β-galactosidase staining*

The senescence status of cells was evaluated by staining for senescence-associated β-galactosidase (SA-β-gal) (Sigma-Aldrich) as previously described (Olivieri, Lazzarini, Babini, et al., 2013). Non-confluent HUVECs cultured in six-well plates were washed twice in phosphate-buffered saline (PBS), pH 7.4, and fixed for 10 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS. After two washes in PBS, they were incubated for 18 h at 37 °C with freshly prepared β-gal staining solution containing 1 mg/mL 5-bromo-4-chloro-3indolyl-β-D-galactopyranoside (Sigma-Aldrich), 5 mM potassium ferrocyanide (Sigma-Aldrich), 150 mM NaCl, 2 mM $MgCl_2$, and 40 mM citric acid titrated with NaH_2PO_4 to pH 6.0. β-Gal was revealed by microscopy, analysing the presence of a blue, insoluble intracellular precipitate. The percentage of β-gal-positive cells was determined by counting at least 500 cells per sample.

2.6. *Quantitative real time PCR (RT-qPCR) of mature microRNAs*

The mirVana isolation kit (Applied Biosystems, Foster City, CA) was used to isolate total RNA (including both microRNA and larger RNA species) from 80×10^3 HUVEC and 1×10^6 U937 cells, following the manufacturer's recommended protocol; RNA was stored at –80 °C until use. Human miR-146a, miR-126 and human RNU44 (reference miRNA) expressions were quantified using the TaqMan MicroRNA assay (Applied Biosystems), as previously described (Olivieri, Lazzarini, Babini, et al., 2013). Briefly, the TaqMan MicroRNA reverse transcription kit was used to reverse transcribe the total RNA; 5 µL of RT mix contained 1 µL of each miR-specific hsa-miR-126 and hsa-miR146a stem-loop primers, 1.67 µL of input RNA, 0.4 µL of 10 mM dNTPs, 0.3 µL of reverse transcriptase, 0.5 µL of 10× buffer, 0.6 µL of RNAse inhibitor diluted 1:10 and 0.55 µL of H_2O. The mixture was incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min. Subsequently RT-qPCR was performed in 20 µL of PCR mix containing 1 µL of 20× TaqMan MicroRNA assay – which in turn contained PCR primers and probes (5′-FAM) – 10 µL of 2× TaqMan Universal PCR Master Mix No Amp Erase UNG (Applied Biosystems), and 5 µL of reverse-transcribed product. The reaction was first incubated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The qRT-PCR was performed on an ABIPRISM 7500 Real Time PCR System (Applied Biosystems). Data were analysed by a 7500 system software (1 1.4.0) with the automatic comparative threshold (Ct) setting for adapting baseline. Detection thresholds were set at 35 Ct. The relative amount of miR-146a and miR-126 was calculated using the Ct method (2):

$$\Delta Ct = Ct(miR146a/miR-126) - Ct(RNU44); 2^{\Delta Ct} \quad (2)$$

Results are expressed in the figures as fold change related to control (CTRL).

2.7. *Quantitative real time PCR (RT-qPCR) of mature mRNA*

Total RNA was extracted as previously using the mirVana isolation kit (Applied Biosystems), and samples were evaluated for nucleic acid quality and quantity using the Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Isolated RNA was used to synthesise cDNA using a reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative polymerase chain reaction real time (RT-qPCR) was performed with the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7500 Real Time PCR System (Applied Biosystems). The primers we used were the one designed by Angel-Morales et al. (2012b) TATA binding protein (TBP) has been used as an endogenous control to determine relative mRNA expression.

The pairs of forward and reverse primers were purchased from Sigma-Aldrich. Product specificity was examined by dissociation curve analysis. The sequences of the primers used are indicated in Table 1. Results are expressed in the figures as fold change related to control (CTRL).

Table 1 – Primers used for quantitative real time PCR (RT-qPCR) analyses.

Gene	Forward primer (5′- 3′)	Reverse primer (5′- 3′)
ICAM-1	TGGCCCTCCATAGACATGTGT	TGGCATCCGTCAGGAAGTG
VCAM-1	ACAGAAGAAGTGGCCCTCCAT	TGGCATCCGTCAGGAAGTG
IL-6	AGGGCTCTTCGGCAAATGTA	GAAGGAATGCCCATTAACAACAA
TNF-a	TGTGTGGCTGCAGGAAGAAC	GCAATTGAAGCACTGGAAAAGG
IRAK-1	CAGACAGGGAAGGGAAACATTTT	CATGAAACCTGACTTGCTTCTGAA
TRAF-6	AGAGTTTGCCGTCCAAGCA	TGGTAGAGGACGGACACAGACA
NFkB	TGGGAATGGTGAGGTCACTCT	TCCTGAACTCCAGCACTCTCTTC
TLR-4	CAGACAGGGAAGGGAAACATTTT	CATGAAACCTGACTTGCTTCTGAA
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA

2.8. Protein extraction and immunoblotting

The cells were incubated on ice for 1 h with the lysis buffer (50 mM Tris, 5 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium vanadate, and 1 mM sodium fluoride, pH 7.4) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA), then lysed with a Sonicator Ultrasonic Liquid Processor XL (Heat System-Ultrasonics, Inc., Newtown CT, NY, USA) and centrifuged at 21,500 g for 10 min at 4 °C to remove detergent-insoluble material. Supernatants were assayed for protein concentration using the Bradford reagent (Sigma-Aldrich). Total protein extracts (15 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Thermo Scientific, Milano, Italy). ColorBurst™ electrophoresis marker (3 μL/gel, Sigma-Aldrich) was used for qualitative molecular mass determinations and for visual confirmation of blot transfer efficiency. Then, blots were blocked with non-fat dry milk in TBS-T (10 mM Tris, 150 mM NaCl, pH 7.6, plus 0.1% Tween-20) and probed overnight at 4 °C with the primary rabbit polyclonal antibody against IRAK-1 (1:1000, Millipore, Merck KGaA, Darmstadt, Germany) and with primary mouse monoclonal antibody against β-actin (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was used as control for protein gel loading. Membranes were washed in TBS-T and incubated for 60 min with the appropriate secondary antibody diluted 1:4000 (Santa Cruz Biotechnology), followed by washing in TBS-T. Proteins were visualised by ECL according to the instructions of the manufacturer (Amersham, Piscataway, NJ, USA).

2.9. Monocyte-endothelial cell adhesion

HUVEC (3×10^5) were dispensed into coverslips (Sigma-Aldrich) positioned into a six-well plate and allowed to achieve semi-confluence. HUVECs were treated with TVV during LPS stimulation as previously described. U937 cells (1×10^6) were added to each well and incubated for 1 h. Non-adherent U937 cell were removed by 3 PBS washes. The coverslips have been removed and placed on glass microscope slides (Sigma-Aldrich). The slides were used to capture images under an optical microscope (using a BX-51 Olympus microscope with a 40× objective). The U937 adherent cells were counted in 3 random fields and expressed as the number of adhered cells per fields related to the number of HUVECs (U937/HUVEC per field).

2.10. Statistical analysis

All measurements were expressed as the mean values plus or minus standard deviation from the mean of at least three independent experiments. The two-tailed paired Student's t-test was used for the analyses. The results were considered significant at the level of $p < 0.05$.

3. Results

3.1. TVV composition

We previously performed some qualitative analyses on the main components of *V. vinifera* L. tendrils extract (TVV) by ESI-MS^n (tandem mass spectrometry) (Fraternale et al., 2015), but in the present study we deeply investigated the TVV quantitative composition to better discuss its biological effects. The detailed phenolic composition of the TVV showed the presence of several types of non-coloured monomeric phenols belonging to diverse phenolic families like phenolic acids (both benzoics and hydroxycinnamics), flavan-3-ols and flavonols (Fig. 1). Up to 18 phenolic compounds were identified and quantified (Table 2) by using the methodology and chromatographic conditions previously described. In qualitative terms, the phenolic profile included 3 benzoic acids (gallic acid, ellagic acid and one ellagic acid derivative), 5 hydroxycinnamic acids (caffeic acid, *t*-caftaric acid, *p*-coumaric acid and one coumaric acid derivative), 1 flavan-3-ol ((+)-catechin) and 9 flavonols (myricetin-3-glucoside, quercetin-3-glucuronide, quercetin-3-rutinoside, quercetin-3-glucoside, isorhamnetin-3-glucoside, myricetin aglucone and three quercetin derivatives). Quantitatively, TVV comprise a rich source of bioactive phenols, mainly flavonols and phenolic acids (16.55 ± 0.21 and 15.88 ± 0.68 mg/g DW, respectively). Most of the flavonols detected corresponded to quercetin and myricetin derivatives, which accounted for 80% and 20% of the total flavonol content, respectively. In particular, quercetin 3-glucuronide was the most abundant flavonol (9.38 ± 0.12 mg/g DW), followed by myricetin 3-glucoside (2.98 ± 0.31 mg/g DW) and quercetin 3-glucoside (2.23 ± 0.04 mg/g DW). In the case of phenolic acids, of especial interest is the high content on benzoic acids (86% of the total phenolic content), mainly due to a major contribution of ellagic acid (6.64 ± 1.71 mg/g DW) and its derivative (5.62 ± 2.30 mg/g DW). In contrast, hydroxycinnamic acids represented minor compounds. Among them, *t*-caftaric and *p*-coumaric acids were the most representative species of this group (0.94 ± 0.11 and 0.50 ± 0.18 mg/g DW, respectively). With regard to flavanols, only (+)-catechin was detected, which accounted for 7% of the total phenolic compounds identified (2.72 ± 0.01 mg/g DW).

3.2. TVV anti-oxidant activity

TVV showed a better anti-oxidant activity than ascorbic acid (with known anti-oxidant properties) at concentrations higher than 10 μg/mL (Fig. 2A), when analysed by DPPH assay. To evaluate the TVV anti-oxidant property into oxidatively injured cultured cells, we decided to induce apoptosis with the H_2O_2 oxidant and analyse the protective effect of TVV on U937 cells. Our results indicate (Fig. 2B and 2C) that TVV alone did not induce any effect on U937 cell death (measured by apoptosis and trypan blue cell viability analyses) at concentrations up to 0.5 mg/mL. When elevated concentrations of TVV are used (0.5–1 mg/mL), an induction of cell death can be observed (near 10% of cell death and up to 30% of apoptosis). But, at lower concentrations, 40–120 μg/mL, a significant moderate protection against H_2O_2 induced apoptosis has been observed (45% TVV treatment vs 55% of H_2O_2 induced apoptosis).

3.3. Anti-inflammatory effect of TVV

In this set of experiments, we used U937 PMA differentiated cells subjected to an acute pro-inflammatory stimulus (1 μg/mL LPS for 6 h) and treated with/without 40 μg/mL TVV. As indicated in Fig. 3A, TLR4, IL-6, TNFα and NF-kB expressions were

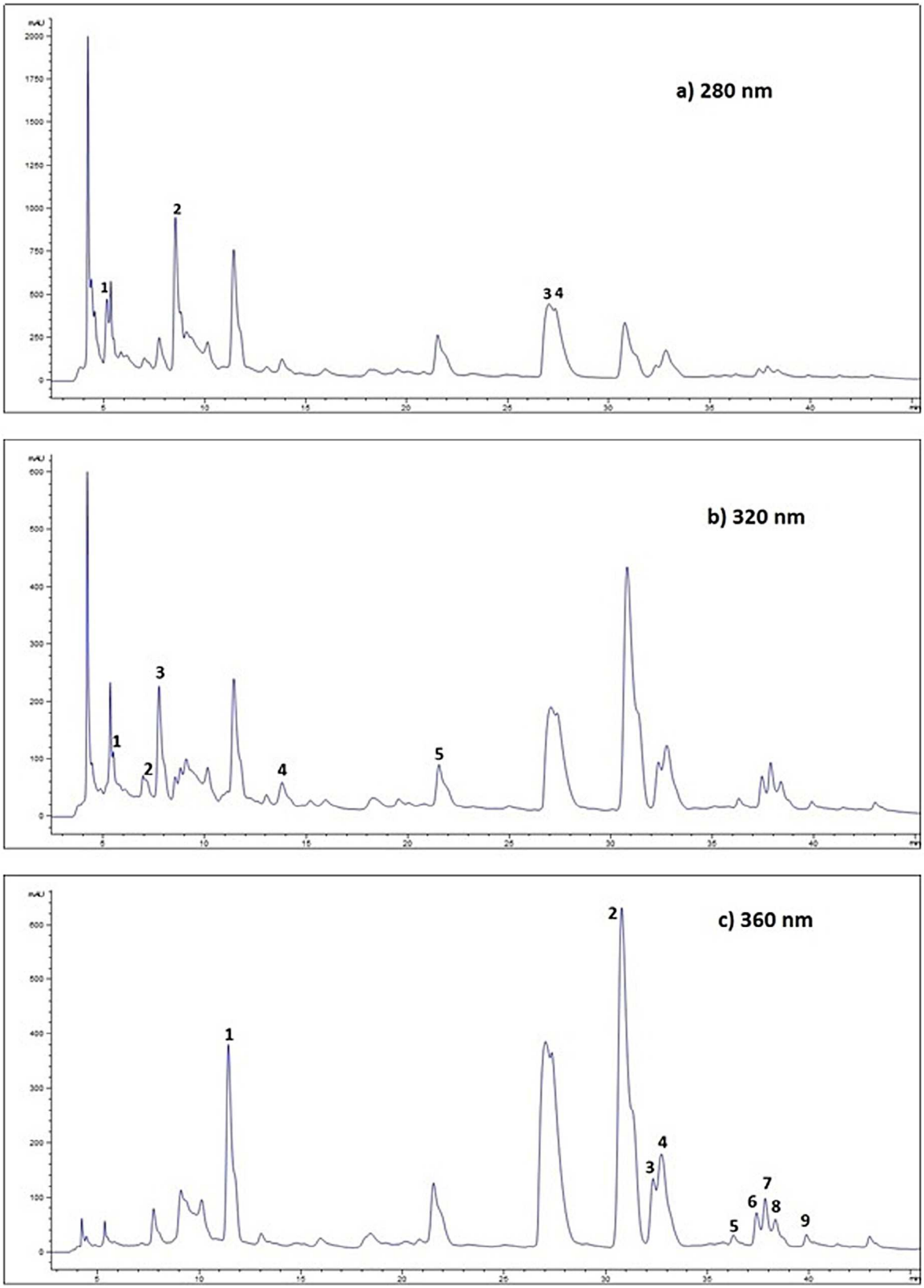

Fig. 1 – HPLC chromatograms recorded at 280 (a), 320 (b), and 360 nm (c) of tendril extract (TVV). Peaks: (a) 1, gallic acid; 2, catechin; 3, ellagic acid; 4, ellagic acid derivative; (b) 1, *p*-coumaric derivative 1; 2, *p*-coumaric derivative 2; 3, *t*-caftaric acid; 4, caffeic acid; 5, *p*-coumaric acid; (c) 1, myricetin 3-O-glucoside; 2, quercetin 3-O-glucuronide; 3, rutin; 4, quercetin 3-O-glucoside; 5, quercetin derivative 1; 6, myricetin; 7, quercetin derivative 2; 8, quercetin derivative 3; 9, isorhamnetin 3-O-glucoside.

Table 2 – Phenolic composition of tendrils extract (TVV). Each value represents mean ± SD (n = 3).

Compound	Phenolic family	λ (nm)	Concentration (mg/g DW)
Gallic acid	Benzoic acid	280	1.44 ± 0.24
Ellagic acid	Benzoic acid	280	6.64 ± 1.71
Ellagic acid derivative	Benzoic acid	280	5.62 ± 2.30
p-Coumaric derivative 1	Hydroxycinnamic acid	320	0.30 ± 0.14
p-Coumaric derivative 2	Hydroxycinnamic acid	320	0.25 ± 0.01
t-Caftaric	Hydroxycinnamic acid	320	0.94 ± 0.11
Caffeic acid	Hydroxycinnamic acid	320	0.17 ± 0.06
p-Coumaric	Hydroxycinnamic acid	320	0.50 ± 0.18
Catechin	Monomeric flavan-3-ol	280	2.73 ± 0.68
Myricetin 3-glucoside	Flavonol	360	2.98 ± 0.31
Quercetin 3-glucuronide	Flavonol	360	9.38 ± 0.12
Quercetin 3-rutinoside (Rutin)	Flavonol	360	0.72 ± 0.06
Quercetin 3-glucoside	Flavonol	360	2.23 ± 0.04
Quercetin derivative 1	Flavonol	360	0.02 ± 0.01
Myricetin	Flavonol	360	0.28 ± 0.02
Quercetin derivative 2	Flavonol	360	0.53 ± 0.04
Quercetin derivative 3	Flavonol	360	0.40 ± 0.01
Isorhamnetin 3-O-glucoside	Flavonol	360	tr.
Sum of benzoic derivatives		280	13.72 ± 0.88
Sum of hydroxycinnamic derivatives		320	2.16 ± 0.02
Sum of flavan-3-ols		280	2.72 ± 0.01
Sum of flavonols		360	16.55 ± 0.21

tr., traces.

reduced by the TVV treatment in LPS-induced inflammation, indicating its anti-inflammatory effect. HUVECs have been already successfully used as a model of cellular senescence by various studies; for this reason, we have chosen HUVECs as a model for evaluating susceptibility to acute inflammation based senescence. The HUVECs cultured until the 13th passage (P13) show 55% of cells positive for β-galactosidase staining assay, indicative of an increased replicative senescence status of the cultured cells compared to the younger ones (10% of cells positive to β-galactosidase staining) (data not shown). Even in this case, we decided to evaluate the effect of TVV during a pro-inflammatory acute stimulus (1 μg/mL LPS for 6 h). As shown in Fig. 3B, even in senescent HUVECs, TLR4, IL-6, TNFα and NF-kB expressions decreased during TVV treatment.

3.4. *Effect of TVV on miR-126 and cell adhesion molecules expression*

In the same way, we wanted to investigate if TVV was able to modulate the expression of miR-126 and its cell adhesion molecules targets (ICAM-1 and VCAM-1). Actually, when miR-126 is down-regulated during inflammation, the expression of its targets (cell adhesion molecules, ICAM-1 and VCAM-1) is enhanced. In our experimental conditions, when an acute pro-inflammatory stimulus has been induced (LPS), we found that TVV (LPS + TVV) significantly increased miR-126 (U937 relative expression LPS vs LPS + TVV: 0.93 ± 0.15 vs 4.5 ± 0.7; $p = 0.008$; and HUVEC relative expression LPS vs LPS + TVV: 171.4 ± 11.7 vs 356 ± 30; $p = 0.01$) and decreased ICAM-1 and VCAM-1

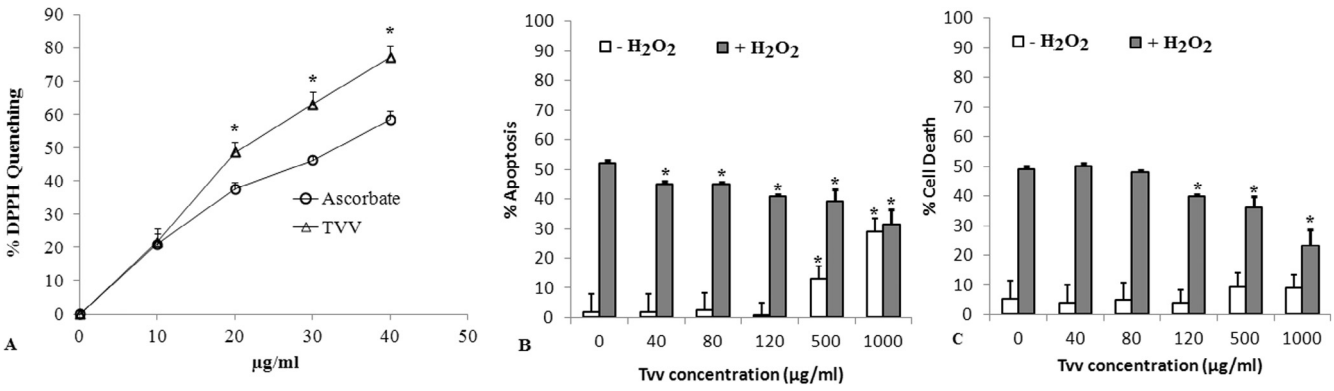

Fig. 2 – Anti-oxidant activity of tendril extract (TVV) at different concentrations (0–40 μg/mL) measured by the DPPH radical assay and H_2O_2 cell death protection. (A) DPPH assay of TVV related to ascorbic acid. (B) TVV protective effect on U937 H_2O_2-induced apoptosis (+H_2O_2). (C) TVV protective effect on U937 H_2O_2-induced cell death (+H_2O_2). Data are the means ± SD of three independent experiments; *$p < 0.05$ related to TVV untreated cells (0 μg/mL), °$p < 0.05$ related to ascorbate.

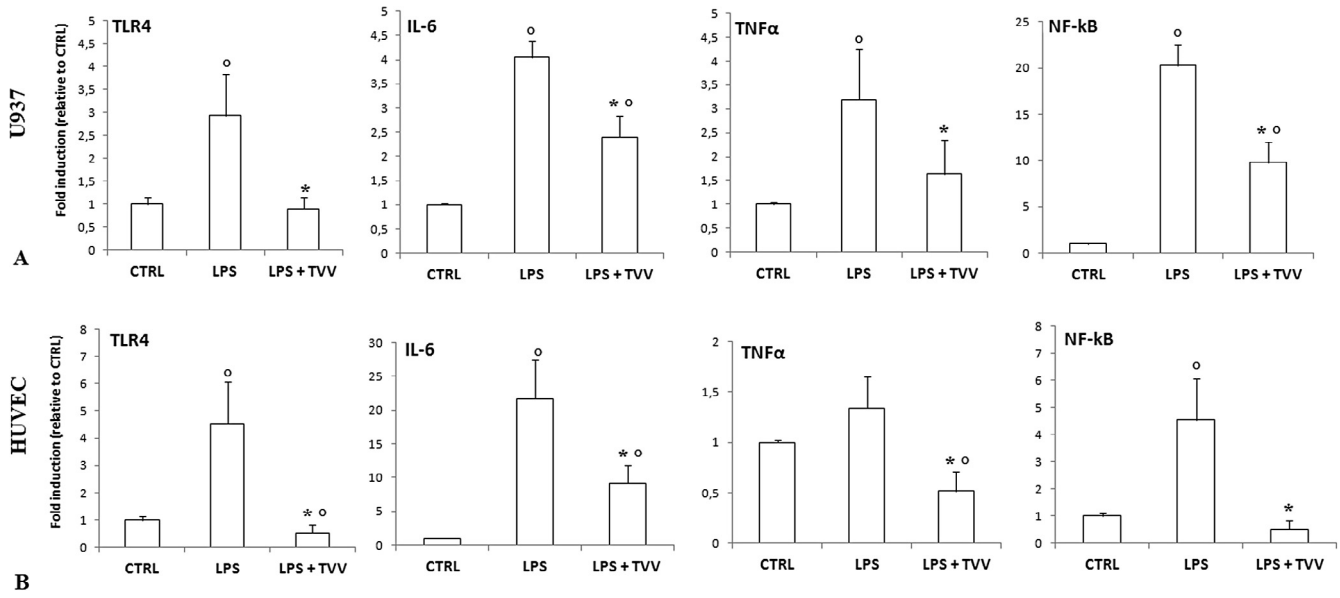

Fig. 3 – Effect of 40 μg/mL tendril extract (TVV) treatment on Toll-like receptor 4 (TLR4) signalling activation and cytokines production. U937 (A) and HUVEC (B) cells were stimulated for 6 h with 1 μg/mL LPS (lipopolysaccharide) to induce a proinflammatory insult. In both cell lines, the mRNA expressions of the receptor (TLR4), NF-kB and the cytokines (IL-6 and TNFα) were decreased after 40 μg/mL TVV treatment (LPS + TVV). Data are reported as fold induction related to untreated control (CTRL) cells. The means ± SD were of at least three independent experiments; °$p < 0.05$ related to CTRL, *$p < 0.05$ related to LPS stimulated cells.

expressions either in U937 and in HUVECs (Fig. 4A and 4B respectively). To confirm our results, the adherence of untreated U937 to HUVEC treated cells (at the same previously described experimental conditions) was determined. Our results identified a marked reduction of adherent U937 when LPS-stimulated HUVECs were treated with TVV as demonstrated by both microscope images (Fig. 4C) and adherent U937 cell count (Fig. 4D).

3.5. *Effect of TVV on miR-146a and its TRAF-6, IRAK-1 targets expression*

A recent study has described a combination of biomarkers related to senescence associated secretory phenotype (SASP) acquisition by HUVECs, which included miR-146a expression, the level of its target protein, IL-1 receptor-associated kinase 1 (IRAK-1), and the amount of IL-6 released into the culture medium; these biomarkers have been called MIRAKIL and used as a model for investigations on senescence-related inflammation (Olivieri, Lazzarini, Babini, et al., 2013). IRAK-1 and TRAF-6 participate to the Toll-like and cytokine receptors signalling, which activates NF-kB. This latter increases miR-146a expression, which in turn inhibits IRAK-1 and TRAF-6 expression as a negative feedback loop (Olivieri, Lazzarini, Babini, et al., 2013). The expression of miR-146a and its targets has been analysed in U937 and HUVECs (Fig. 5). The expression of U937 miR-146a was elevated in LPS-stimulated cells and even increased during TVV treatment, while in HUVEC senescent cells its expression increased only during TVV treatment (LPS + TVV) (U937 relative expression LPS vs LPS + TVV: 0.76 ± 0.008 vs 2.93 ± 0.33; $p = 0.01$; and HUVEC relative expression LPS vs LPS + TVV: 0.19 ± 0.006 vs 3.23 ± 0.4; $p = 0.007$). Since miR-146a targets TRAF-6 and IRAK-1, we evaluated their mRNA expression. The LPS stimulation induced a slight increase that was prevented during TVV treatment (LPS + TVV) in both cell lines (Fig. 5A and 5C). During acute U937 LPS-stimulation, miR-146a is up-regulated but IRAK-1 expression was unchanged (Fig. 5B). Interestingly, when TVV induced a greater increase of miR-146a, a decrease of the IRAK-1 mRNA and protein expression can be observed. Furthermore, either in the CTRL and LPS + TVV HUVEC treated cells, IRAK-1 protein expression presented a double band, while only one band was present during LPS stimulation alone in HUVECs (Fig. 5D).

4. Discussion

The present study investigated the tendril extract of *V. vinifera* L. (TVV) for its composition and biological effects. Flavonoids, polyphenols and anthocyanins have shown to be the major constituents of TVV extract. We used mass spectrometry to obtain more qualitative information on the main components of TVV extract and found the presence of different class of compounds: flavonols (rutin, quercetin-3-glucuronide), phenolic acids (caffeic, ellagic and caftaric acids), fumaric and citric acids. Most of these compounds have been described to have anti-oxidant and/or anti-inflammatory properties. The ellagic acid has already been demonstrated to have an intestinal anti-inflammatory action (Marin, Giner, Rios, & Recio, 2013), a positive effect on lipopolysaccharide-induced inflammation (Lee et al., 2014), and is able to inhibit IL-1b-induced cell adhesion molecule expression in HUVEC cells (Yu, Wang, Liu, & Chen, 2007). Rutin (quercetin 3-rutinoside) has

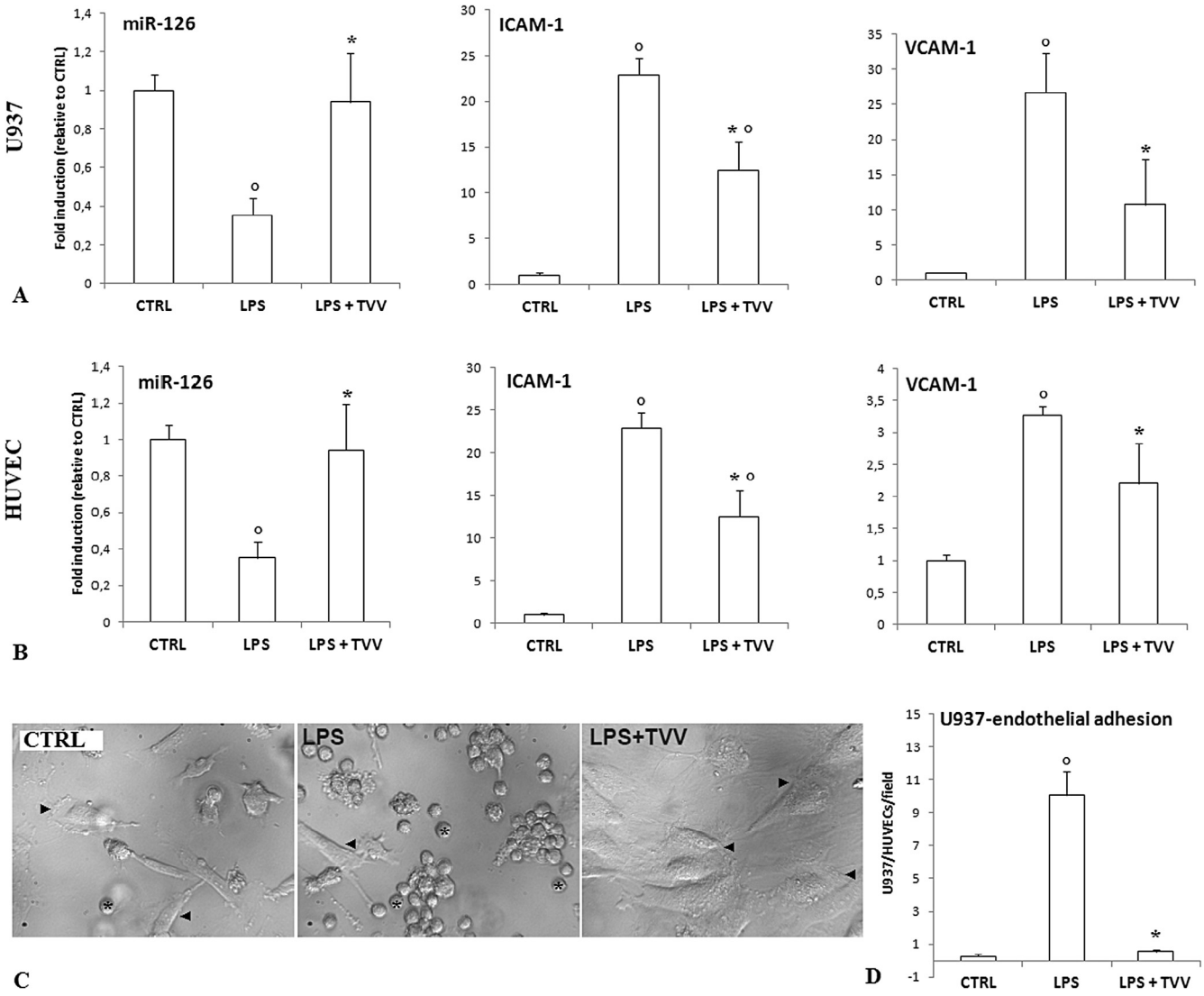

Fig. 4 – Effect of 40 μg/mL tendril extract (TVV) treatment on miR-126 and its cell adhesion molecules targets (ICAM-1 and VCAM-1) expression in LPS (lipopolysaccharide) stimulated cells. In both cell lines, U937 (A) and HUVEC (B), the LPS down-regulation of miR-126 (LPS) was restored during TVV treatment (LPS + TVV). TVV showed a decresed expression of both ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion protein 1) miR-126 targets. U937-endothelial adhesion (C) was evaluated adding untreated U937 cells to LPS-stimulated HUVECs without/with 40 μg/mL TVV (LPS and LPS + TVV respectively). Typical images captured on a BX-51 Olympus microscope with a 40× objective of U937 adherent to HUVECs are shown. The images were employed to evaluate the number (D) of adherent U937 (*) cells related to HUVECs (◀) present in each field analysed (3 fields for each treatment conditions of 3 independent experiments). RT-qPCR values are reported as fold induction related to CTRL. Data are the means ± SD of three independent experiments; °$p < 0.05$ related to CTRL, *$p < 0.05$ related to LPS stimulated cells.

been revealed to suppress the production of tumour necrosis factor-α (TNF-α) and activation of nuclear factor-κB (NF-κB) by LPS in HUVECs (Lee, Ku, & Bae, 2012). Quercetin can exhibit anti-inflammatory properties, but its metabolite, quercetin-3-glucuronide, does not seem to have the same property (Milenkovic et al., 2013). Other authors evaluated the anti-oxidant activity of plant extracts containing the same compounds that we found in our TVV (Vlase et al., 2014). The presence of these anti-oxidant compounds in TVV extract is likely responsible for its anti-oxidant activity. In fact, the results of the DPPH radical assay have shown better anti-oxidant properties of TVV than ascorbic acid. Furthermore, TVV has shown a cytoprotective effect in U937 cells pretreated with H_2O_2, showing a significant apoptosis inhibition at low concentrations of TVV (40 μg/mL) after 5 hours of treatment. Since TVV at low concentrations demonstrated an anti-oxidant activity, we decided to employ 40 μg/mL concentration in our cell treatments to investigate its possible anti-inflammatory effect.

It has already been demonstrated that anti-oxidants regulate NF-kB activation and inflammatory genes in LPS stimulated

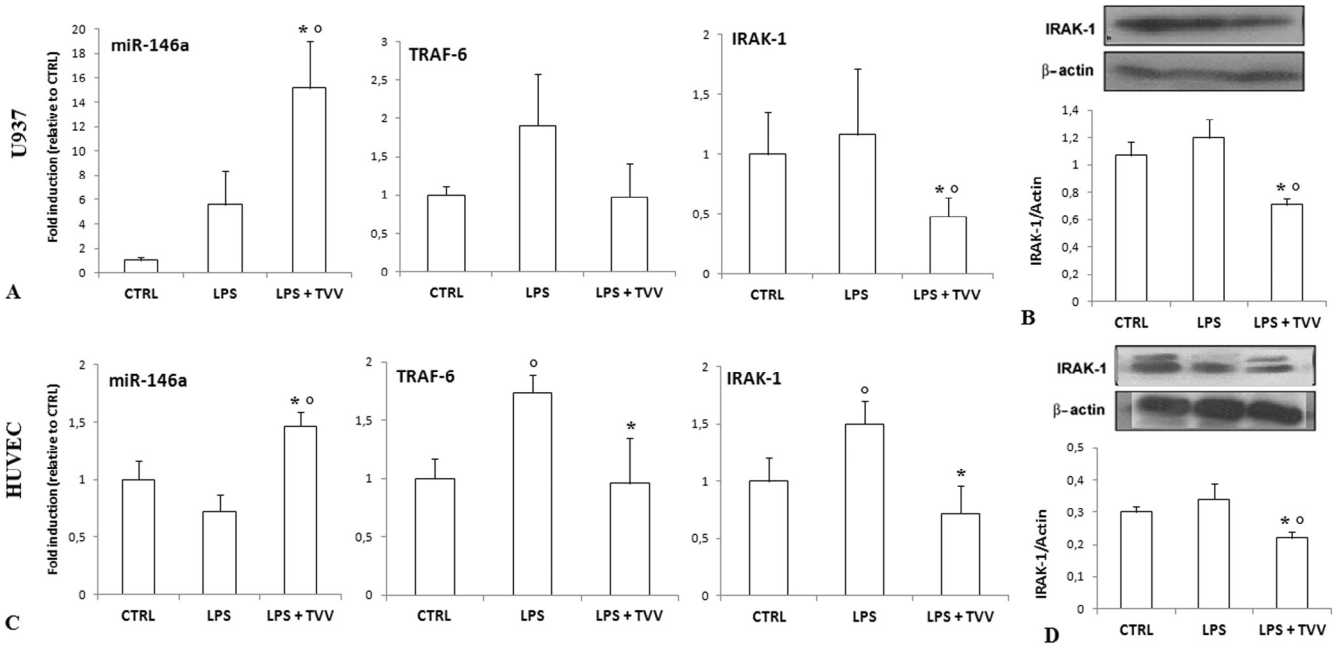

Fig. 5 – Effect of 40 μg/mL tendril extract (TVV) treatment on miR-146a and its IRAK-1/TRAF6 (IL-1 receptor-associated kinase 1/TNF receptor-associated factor 6) targets expression in LPS-stimulated cells. In U937 cells (A) we can observe a LPS (lipopolysaccharide) miR-146a up-regulation (LPS) that increased after TVV treatment (LPS + TVV). The expression of TRAF-6 and IRAK-1 targets during LPS stimulus (LPS) decreased after TVV treatment (LPS + TVV). In the same way, IRAK-1 protein expression (B) decreased in LPS-stimulated U937 cells during TVV treatment. In HUVEC senescent cells (C), miR-146a was up-regulated in the presence of TVV (LPS + TVV). Again, the expression of TRAF-6 and IRAK-1 targets during LPS stimulus (LPS) decreased after TVV treatment (LPS + TVV). IRAK-1 protein expression (D) decreased in LPS-stimulated U937 cells during TVV treatment. Similar IRAK-1 protein pattern in CTRL and LPS + TVV samples are shown. RT-qPCR values are reported as fold induction related to untreated control (CTRL) cells. Data are the means ± SD of three independent experiments; °$p < 0.05$ related to CTRL, *$p < 0.05$ related to LPS stimulated cells.

macrophages (Kim et al., 2008); for this reason, we decided to investigate how TVV could influence NF-kB-regulated inflammatory genes via the TLR4 signalling. TLR4 is a member of the Toll family of pattern-recognition receptors, which can be stimulated by LPS. In this study, we observed a decreased expression of TLR4, IL6, TNFα and NF-kB after LPS stimulation either in U937 and senescent HUVEC cells during TVV treatment. Furthermore, since NF-kB stimulates the expression of cell adhesion molecules (ICAM-1 and VCAM-1), we wanted to investigate if TVV was able to modulate the expression of miR-126 and its cell adhesion molecules targets. Recently, flavonoid-rich extracts have been found to regulate microRNA (miRNA) expression. MiRNA are noncoding RNAs able to regulate gene expression responsible of both physiological and pathological processes such as inflammation (Olivieri, Lazzarini, Babini, et al., 2013). Previous studies demonstrated that red wine polyphenols showed a protection against ROS production accompanied by the reduction of the NF-kB activity and related gene expression such as VCAM-1. Furthermore, the same authors also found that the anti-inflammatory property was mediated by the induction of miR-126 (Angel-Morales et al., 2012a). In the same way, we found that TVV is able to induce miR-126 expression, and to reduce cell adhesion molecules expression (either ICAM-1 or VCAM-1) and U937 adhesion to HUVECs during LPS stimulation.

The MIRAKIL biomarkers have been used as a model for investigations on senescence-related inflammation. HUVECs acquire the senescence-associated secretory phenotype (SASP) by modulating miR-146a expression, the level of its target protein IRAK-1 (IL-1 receptor-associated kinase 1), and the amount of IL-6 released into the culture medium. IRAK-1 and TRAF-6 (TNF receptor-associated factor 6) are components of the Toll-like and cytokine receptors signalling that activate NF-kB. This latter up-regulates miR-146a, which in turn inhibits IRAK-1 and TRAF-6 expression as a negative feedback loop (Olivieri, Lazzarini, Recchioni, et al., 2013). Following TLR4 stimulation, IRAK-1 is engaged to the TLR4 complex where it is phosphorylated by IRAK4 promoting autophosphorylating activity of IRAK-1. At this time, IRAK-1 is hyper-phosphorylated and can detach from the receptor complex if associated with TRAF6. These processes are responsible for the activation of NF-kB. Importantly, the phosphorylation of IRAK-1 induces a transient appearance of higher molecular weight forms and subsequent loss of the protein (Dunne et al., 2010). Our results indicate that during an acute LPS stimulation, miR-146a increases in U937 with TVV treatment but not in HUVECs,

probably because of the senescence phenotype. MiR-146a decreases TRAF6 and IRAK-1 expression targets. In both U937 and HUVECs, IRAK-1 protein expression decreases during TVV treatment in LPS-stimulated cells, and in HUVECs the protein pattern remains similar to that of control (CTRL) cells. Actually, as observed in HUVECs, either in CTRL or TVV LPS-stimulated cells, the phosphorylated IRAK-1 forms are similar. Taking together all these results, we believe that during LPS acute stimulation, TLR4 signalling stimulates cytokine (IL-6 and TNFα) and cell adhesion molecules (ICAM-1 and VCAM-1) transcription by inducing NF-kB expression. This latter is also responsible for the miR-146a up-regulation. In this way, IRAK-1 has been degraded after its activation. During TVV treatment, the LPS stimulation can be prevented, inhibiting TLR4 signalling, cytokine production and cell adhesion. In addition, the increased miR-146a expression, with a negative feedback loop mechanism, inhibits TRAF6 and IRAK-1 transcription, avoiding IRAK-1 receptor detachment and activation.

5. Conclusion

The grapevine has been investigated for fruit and leaf properties (Xia, Deng, Guo, & Li, 2010), but to our knowledge we have shown for the first time that a grape tendril extract has anti-inflammatory effect involving miRNA modulation. The antioxidant, cytoprotective and antiinflammatory properties of TVV could be of great interest as grape tendrils are used for dietary purposes. Further studies are needed for the elucidation of the molecular basics of the TVV anti-inflammatory properties, but it can be speculated, in concordance with data from literature, that most probably the polyphenols in the TVV extract could be responsible for the benefits observed. Its positive effect on endothelial inflammation and its general anti-oxidant properties could probably make the grape tendril extract an option for functional foods for atherosclerosis and other vascular diseases, providing benefits to health.

Conflict of interest

The authors declare that they have no conflicts of interest. This article does not contain any studies with human or animal subjects.

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REFERENCES

Angel-Morales, G., Noratto, G., & Mertens-Talcott, S. (2012a). Red wine polyphenolics reduce the expression of inflammation markers in human colon-derived CCD-18Co myofibroblast cells: Potential role of microRNA-126. *Food & Function*, 3, 745–752.

Angel-Morales, G., Noratto, G., & Mertens-Talcott, S. (2012b). Standardized curcuminoid extract (*Curcuma longa* L.) decreases gene expression related to inflammation and interacts with associated microRNAs in human umbilical vein endothelial cells (HUVEC). *Food & Function*, 3, 1286–1293.

Bouhamidi, R., Prévost, V., & Nouvelot, A. (1998). High protection by grape seed proanthocyanidins (GSPC) of polyunsaturated fatty acids against UV-C induced peroxidation. *Comptes Rendus de l'Académie des Sciences – Series III – Sciences de la Vie*, 321, 31–38.

Calonje, M., Cubas, P., Martìnez-Zapater, J. M., & Carmona, M. J. (2007). Floral meristem identity genes are expressed during development in grapevine. *Plant Physiology*, 135, 1491–1501.

Colussi, C., Albertini, M. C., Coppola, S., Rovidati, S., Galli, F., & Ghibelli, L. (2000). H_2O_2-induced block of glycolysis as an active ADP-ribosylation reaction protecting cells from apoptosis. *FASEB Journal*, 14, 2266–2276.

Cuendet, M., Hostettmann, K., Potterat, O., & Dyatmiko, W. (1997). Iridoid glucosides with free radical scavenging properties from *Fagraea blumei*. *Helvetica Chimica Acta*, 80, 1144–5112.

Dani, C., Oliboni, L. S., Agostini, F., Funchal, C., Serafini, L., Henriques, J. A., & Salvador, M. (2010). Phenolic content of grapevine leaves (*Vitis labrusca* var. Bordo) and its neuroprotective effect against peroxide damage. *Toxicology in Vitro*, 24, 148–153.

Dunne, A., Carpenter, S., Brikos, C., Gray, P., Strelow, A., Wesche, H., Morrice, N., & O'Neill, L. A. (2010). IRAK-1 and IRAK4 promote phosphorylation, ubiquitination, and degradation of MyD88 adaptor-like (Mal). *The Journal of Biological Chemistry*, 285, 18276–18282.

Fraternale, D., De Bellis, R., Calcabrini, C., Potenza, L., Cucchiarini, L., Mancini, U., Dachà, M., & Ricci, D. (2011). Aqueous extract from *Vitis vinifera* tendrils is able to enrich keratinocyte antioxidant defences. *Natural Product Communications*, 6, 1315–1319.

Fraternale, D., Ricci, D., Verardo, G., Gorassini, A., Stocchi, V., & Sestili, P. (2015). Antifungal activity of *Vitis vinifera* tendrils extract against phytopathogenic fungi. *Natural Product Communications*, 10, 1037–1042.

Gordillo, B., Cejiudo-Bastante, M. J., Rodriguez-Pulido, F. J., Jara-Palacios, M. J., Ramirez-Perez, P., Gonzalez-Miret, M. L., & Heredia, F. (2014). Impact of adding white pomace to red grapes on the phenolic composition and color stability of syrah wines from a warm climate. *Journal of Agricultural and Food Chemistry*, 62, 2663–2671.

Harman, D. (2006). Free radical theory of aging: An update: Increasing the functional life span. *Annals of the New York Academy of Sciences*, 1067, 10–21.

Kim, J. H., Na, H. J., Kim, C. K., Kim, J. Y., Ha, K. S., Lee, H., Chung, H. T., Kwon, H. J., Kwon, Y. G., & Kim, Y. M. (2008). The non-provitamin A carotenoid, lutein, inhibits NF-kappaB-dependent gene expression through redox-based regulation of the phosphatidylinositol 3-kinase/PTEN/Akt and NF-kappaB-inducing kinase pathways: Role of H_2O_2 in NF-kappaB activation. *Free Radical Biology and Medicine*, 45, 885–896.

Lee, J., Kim, S., Namgung, H., Jo, Y. H., Bao, C., Choi, H. K., Auh, J. H., & Lee, H. J. (2014). Ellagic acid identified through metabolomic analysis is an active metabolite in strawberry ('seolhyang') regulating lipopolysaccharide-induced

inflammation. *Journal of Agricultural and Food Chemistry*, *62*, 3954–3962.

Lee, W., Ku, S. K., & Bae, J. S. (2012). Barrier protective effects of rutin in LPS-induced inflammation *in vitro* and *in vivo*. *Food and Chemical Toxicology*, *50*, 3048–3055.

Marin, M., Giner, R. M., Rios, J. L., & Recio, M. C. (2013). Intestinal anti-inflammatory activity of acute and chronic ellagic acid in the dextrane sulfate sodium models of mice colitis. *Journal of Ethnopharmacology*, *150*, 925–934.

Milenkovic, D., Jude, B., & Morand, C. (2013). miRNA as molecular target of polyphenols underlying their biological effects. *Free Radical Biology and Medicine*, *64*, 40–51.

Noratto, G. D., Angel-Morales, G., Talcott, S. T., & Mertens-Talcott, S. U. (2011). Polyphenolics from acaí (*Euterpe oleracea* Mart.) and red muscadine grape (*Vitis rotundifolia*) protect human umbilical vascular Endothelial cells (HUVEC) from glucose- and lipopolysaccharide (LPS)-induced inflammation and target microRNA-126. *Journal of Agricultural and Food Chemistry*, *59*, 7999–8012.

Noratto, G. D., Kim, Y., Talcott, S. T., & Mertens-Talcott, S. U. (2011). Flavonol-rich fractions of yaupon holly leaves *(Ilex vomitoria*, Aquifoliaceae) induce microRNA-146a and have anti-inflammatory and chemopreventive effects in intestinal myofibroblast CCD-18Co cells. *Fitoterapia*, *82*, 557–569.

Olivieri, F., Lazzarini, R., Babini, L., Prattichizzo, F., Rippo, M. R., Tiano, L., Di Nuzzo, S., Graciotti, L., Festa, R., Brugè, F., Orlando, P., Silvestri, S., Capri, M., Palma, L., Magnani, M., Franceschi, C., Littarru, G. P., & Procopio, A. D. (2013). Anti-inflammatory effect of ubiquinol-10 on young and senescent endothelial cells via miR-146a modulation. *Free Radical Biology and Medicine*, *63*, 410–420.

Olivieri, F., Lazzarini, R., Recchioni, R., Marcheselli, F., Rippo, M. R., Di Nuzzo, S., Albertini, M. C., Graciotti, L., Babini, L., Mariotti, S., Spada, G., Abbatecola, A. M., Antonicelli, R., Franceschi, C., & Procopio, A. D. (2013). MiR-146a as marker of senescence-associated proinflammatory status in cells involved in vascular remodeling. *Age*, *35*, 1157–1172.

Salminen, A., Ojala, J., Kaarniranta, K., & Kauppinen, A. (2012). Mitochondrial dysfunction and oxidative stress activate inflammasomes: Impact on the aging process and age-related diseases. *Cellular and Molecular Life Sciences*, *69*, 2999–3013.

Tome-Carneiro, J., Gonzalvez, M., Larrosa, M., Yanez-Gascon, M. J., Garcia-Almagro, F. J., Ruiz Ros, J. A., Tomas-Barberan, F. A., Garcia-Conesa, M. T., & Espin, J. C. (2013). Grape resveratrol increases serum adiponectin and downregulates inflammatory genes in peripheral blood mononuclear cells: A triple-blind, placebo-controlled, one-year clinical trial in patients with stable coronary artery disease. *Cardiovascular Drugs and Therapy*, *27*, 37–48.

Tome-Carneiro, J., Larrosa, M., Yanez-Gascon, M. J., Davalos, A., Gil-Zamorano, J., Gonzalvez, M., Garcia-Almagro, F. J., Ruiz Ros, J. A., Tomas-Barberan, F. A., Espin, J. C., & Garcia-Conesa, M. T. (2013). One-year supplementation with a grape extract containing resveratrol modulates inflammatory-related microRNAs and cytokines expression in peripheral blood mononuclear cells of type 2 diabetes and hypertensive patients with coronary artery disease. *Pharmacological Research*, *72*, 69–82.

Ulusoy, S., Ozchan, G., Yucesan, F. B., Ersoz, S., Orem, A., Alkanat, M., Yulug, E., Kaynar, K., & Al, S. (2012). Anti-apoptotic and anti-oxidant effects of grape seed proanthocyanidin extract in preventing cyclosporine A-induced nephropathy. *Nephrology*, *17*, 372–379.

Vlase, L., Benedec, D., Hanganu, D., Damian, G., Csillag, I., Sevastre, B., Mot, A. C., Silaghi-Dumitrescu, R., & Tilea, I. (2014). Evaluation of antioxidant and antimicrobial activities and phenolic profile for *Hyssopus officinalis*, *Ocimum basilicum* and *Teucrium chamaedrys*. *Molecules*, *19*, 5490–5507.

Xia, E. Q., Deng, G. F., Guo, Y. J., & Li, H. B. (2010). Biological activities of polyphenols from grapes. *International Journal of Molecular Sciences*, *11*, 622–646.

Yu, Y. M., Wang, Z. H., Liu, C. H., & Chen, C. S. (2007). Ellagic acid inhibits IL-1b-induced cell adhesion molecule expression in human umbilical vein endothelial cells. *British Journal of Nutrition*, *97*, 692–698.