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ARTICLE

In Vitro Protective Effects of Two Extracts from Bergamot Peels on Human Endothelial Cells Exposed to Tumor Necrosis Factor-α (TNF-α)

Domenico Trombetta,[†] Francesco Cimino,[†] Mariateresa Cristani,[†] Giuseppina Mandalari,[†] Antonella Saija,*[,†] Giovanna Ginestra,[†] Antonio Speciale,[†] Joselita Chirafisi,[†] Giuseppe Bisignano,[†] Keith Waldron,[‡] Arjan Narbad,[§] and Craig B. Faulds[‡]

[†]Department Farmaco-Biologico, School of Pharmacy, University of Messina, Viale Annunziata, 98168 Messina, Italy, [‡]Sustainability of the Food Chain Exploitation Platform, and [§]Integrated Biology GI Tract Programme, Institute of Food Research, Colney Lane, Norwich NR4 7UA, United Kingdom

Bergamot (*Citrus bergamia* Risso) is a less commercialized *Citrus* fruit, mainly used for its essential oil extracted from the peel. Bergamot peel (BP) represents about 60% of the processed fruits and is regarded as primary waste. However, it contains good amounts of useful compounds, such as pectins and flavonoids. Many of the bioactivities of *Citrus* flavonoids appear to impact vascular endothelial cells. Herein, we report the protective effect of two flavonoid-rich extracts from BP (endowed with radical-scavenging properties and lacking genotoxic activity) against alterations in cell modifications induced by the pleiotropic inflammatory cytokine tumor necrosis factor-α (TNF-α) on human umbilical vein endothelial cells (HUVECs), as demonstrated by monitoring intracellular levels of malondialdehyde/4-hydroxynonenal, reduced and oxidized glutathione and superoxide dismutase activity, and the activation status of nuclear factor-κB (NF-κB). Thus, BP appears to be a potential source of natural antioxidant/anti-inflammatory phytocomplexes to be employed as ingredients of nutraceutical products or functional foods.

KEYWORDS: Bergamot peels; human vein endothelial cells; TNF-α; SOS chromotest; antioxidant

INTRODUCTION

The vascular endothelial cell is a preferential target for therapy in various pathological conditions, including cardiovascular disease, neurodegenerative disease, and cancer, all of which underlie alterations of this cell type. Vascular endothelial dysfunction or injury impair endothelial functions and may be induced by aging, smoking, inflammation, trauma, hyperlipidaemia, or hyperglycaemia, which are in fact among a myriad of risk factors that may contribute to the pathogenesis of many cardiovascular and degenerative diseases.

Oxidative stress and inflammation are the prominent pathways of vascular endothelial dysfunction in many pathophysiological conditions. Oxidative stress is mainly caused by an imbalance between the activity of endogenous pro-oxidative (such as NADPH oxidase, xanthine oxidase, or the mitochondrial respiratory chain) and antioxidant [such as superoxide dismutase (SOD), glutathione peroxidase, heme oxygenase, thioredoxin peroxidase/peroxiredoxin, catalase, and paraoxonase] enzymes. In addition, small-molecular-weight antioxidants might have a role in defense against oxidative stress. The production of reactive oxygen species (ROS) can stimulate the cytokine cascade through nuclear factor-κB (NF-κB)-induced transcriptional events, which then induce the expression of the inflammatory cytokine tumor necrosis factor-α (TNF-α) (*1*, *2*). Accumulating evidence suggests that TNF-α in turn plays a pivotal role in the disruption of endothelial functions, as well as increasing, through several mechanisms, ROS production and, thus, significantly contributing to the maintenance of the oxidant-rich environment at the inflammatory locus. In particular, TNF-α stimulates $O_2^{\bullet -}$ production in endothelial cells via ceramide-activated protein kinase (CAPK), NADPH oxidase (*3*), XO (*4*), etc. Several studies have demonstrated that TNF-α induces an increased production of ROS inside the mitochondrion; in fact, TNF-α-induced mitochondrial ROS were shown to be tightly correlated with cytotoxicity, and ROS formation could be effectively scavenged by the mitochondrial glutathione system (*2*).

Bergamot (*Citrus bergamia* Risso) is a less commercialized *Citrus* fruit typical of the Reggio Calabria province in southern Italy, where it is mainly used for its essential oil, extracted from the peel. Bergamot peel (BP) represents about 60% of the processed fruits and is regarded as primary waste; if not processed further, it may cause environmental problems because of its fermentability. However, as well as other kinds of *Citrus* peel wastes (*5*), very useful compounds, such as pectins and flavonoids, have been found in BP (*6*). BP contains the characteristic

*To whom correspondence should be addressed: Department Farmaco-Biologico, School of Pharmacy, University of Messina, Viale Annunziata, 98168 Messina, Italy. Telephone: 0039-90-6766480. Fax: 0039-90-6766474. E-mail: saija@pharma.unime.it.

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Citrus flavanone rutinosides and neo-hesperosides derived from naringenin, eriodictyol, and hesperetin and small amounts of some flavone *O*- and *C*-glycosides not previously found in orange and lemon peels.

Much of the activity of *Citrus* flavonoids appears to impact blood and microvascular endothelial cells, and it is not surprising that major areas of research on the biological actions of *Citrus* flavonoids have been cardiovascular disease and inflammatory pathologies (*7*). The aim of the present study was to evaluate if two flavonoid-rich extracts from BP, previously characterized for their chemical profile (*6*) and tested for their radical scavenger activity, are able *in vitro* to protect human vessel endothelial cells against alterations in intracellular redox status and oxidative damages induced by TNF-α. With this target, we monitored, as hallmarks of oxidative damage induced by TNF-α on human umbilical vein endothelial cells (HUVECs), the intracellular levels of malondialdehyde/4-hydroxynonenal (MDA/HNE) of reduced and oxidized glutathione (GSH and GSSG) and SOD activity and the activation status of NF-κB. To clarify the mechanisms involved in their protective activity in a better way, these extracts were examined, by means of an *in vitro* model on human whole blood, for their capability to inhibit cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) activity. Finally, because under certain conditions plant polyphenols are reported to act as mutagens and pro-oxidants, the extracts were tested for the genotoxic effect by means of the SOS chromotest on the *Escherichia coli* PQ37 strain.

MATERIALS AND METHODS

Reagents. Methanol, ethanol, and acetonitrile were obtained from CarloErba Reagent (Milan, Italy) in their highest commercially available purity grade. All other reagents were purchased from Sigma-Aldrich (Milan, Italy), unless otherwise specified.

Extract Preparations. BP (a mix from the three major cultivars Fantastico, Femminello, and Castagnaro) was obtained from a bergamot processing factory (Consorzio del Bergamotto) in southern Italy. An alcohol-insoluble residue was prepared as previously described (*6*) and then used to obtain four liquid fractions [each by sequential ethanolic fractionation by two 70% (v/v) extractions followed by two 100% (v/v) ethanol extractions], called 70 E1, 70 E2, 100 E1, and 100 E2 (100 g of peel extracted yielding 970 mL of 70 E1, 1000 mL of 70 E2, 980 mL of 100 E1, and 970 mL of 100 E2). The composition of these extracts (sugars, uronic acid, simple phenolics, flavonoids, and psoralens) was confirmed in our experiments (*6*). Briefly, the extracts were analyzed using a Phenomenex Luna C18 reverse-phase column (250 × 4.6 mm, 5 μm; Phenomenex, Macclesfield, U.K.) in combination with an Agilent HP1100 HPLC instrument (Agilent Ltd., West Lothian, U.K.) with a diode array detector. Specific UV−vis data were collected at 220, 270, 325, 370, and 520 nm (with overall data collected between 200 and 600 nm). The main components of these extracts were naringin, neoeriocitrin, neohesperidin, hesperetin monorhamnoside, and naringenin monorhamnoside, in addition to other minor flavones (apigenin, luteolin, and diosmetin-derived), flavanones (eriodictyol, naringenin, and hesperetin-derived), and very low levels of psoralens and lipophilic phenolics. All known compounds were measured directly using standard curves obtained by running commercial standards of previously identified bergamot and *Citrus* phytochemicals and other common plant phenolics and various flavonoids.

Previously, in *in vitro* studies (*8*), it has been shown that, among the different four fractions, 100 E1 and 70 E2 have the best antimicrobial activity. These data were also consistent with those obtained in a preliminary series of experiments concerning the antioxidant activity of these four extracts, as measured by the Folin−Ciocalteau assay. With this background, in the present study, only the extracts 100 E1 and 70 E2 were employed and their flavonoid profile is shown in **Table 1**.

Radical Scavenger Activity. *Folin−Ciocalteau Assay*. The amount of total phenols in the extracts were determined according to the Folin−Ciocalteu colorimetric method (*9*) and expressed as gallic acid equivalents (μg/mg). Each determination was performed in triplicate and repeated at least 3 times.

Table 1. Flavonoid Profile of the BP Extracts 70 E2 and 100 E1[a]

	70 E2 (μg/g of dry extract)	100 E1 (μg/g of dry extract)
apigenin 6,8-di-*C*-glucoside	449.30 ± 0.58	144.23 ± 0.08
apigenin monoglc/monorha[b]	1144.95 ± 0.42	476.24 ± 0.08
diosmetin 6,8-di-*C*-glucoside	268.04 ± 0.15	105.67 ± 0.05
diosmetin monoglucoside	640.33 ± 0.12	330,67 ± 0.035
diosmetin monorhamnoside	267.73 ± 0.09	208.34 ± 0.065
eriocitrin	415.37 ± 0.91	0
eriodictyol monorhamnoside	1329.90 ± 1.05	412.68 ± 0.21
hesperetin monorhamnoside	4796.91 ± 1.10	2114.77 ± 0.88
luteolin monoglc/monorha	721.96 ± 0.21	201.34 ± 0.052
narirutin	835.67 ± 0.98	0
naringin	15753.51 ± 1.25	6375.84 ± 1.34
naringenin monorhamnoside	2518.46 ± 0.97	1341.61 ± 0.95
neoeriocitrin	12365.52 ± 1.15	3759.73 ± 1.31
neohesperidin	7525.77 ± 1.01	2784.56 ± 1.09

[a] Data are expressed as mean ± SD of three analyses. [b] monoglc/monorha = monoglucoside/monorhamnoside.

DPPH Test. The free-radical-scavenging capacity of the extracts was tested as bleaching of the stable radical diphenylpicrylhydrazyl (DPPH) (*9*). The reaction mixture (3.5 mL of methanol) contained 100 mM DPPH and different concentrations of BP extracts; an equal volume of the solvent employed to dissolve the extracts (37.5 μL) was added to control tubes. After 20 min at room temperature, the absorbance was recorded at 517 nm. All experiments were carried out in triplicate. Results were expressed as a percentage decrease with respect to control values; mean scavenging concentrations (SC_{50}) and 95% confidence limits (95% CL) were calculated using the Litchfield and Wilcoxon test.

In the Folin−Ciocalteau assay and DPPH test, the antioxidant/radical scavenging activity of the extracts under investigation was compared to that of Trolox.

Genotoxic Activity in the SOS Chromotest. The genotoxic activity of the bergamot extracts was studied in the SOS chromotest, which employs the error-prone DNA repair pathway of *E. coli* PQ37, also known as the SOS response, a complex regulatory network that is induced by DNA-damaging substances. The test involves incubation of the bacteria with the sample under investigation and subsequent determination of β-galactosidase (β-gal) activity, because β-gal synthesis is used as a measure of SOS repair system induction. The activity of the constitutive enzyme alkaline phosphatase (AP) was used as a measure of protein synthesis and toxicity. Briefly, an exponential-phase culture grown to OD_{600} = 0.4 in Luria Bertani (LB) medium plus ampicillin at 37 °C was diluted 1:10 in fresh medium. Fractions (0.6 mL) were distributed into glass test tubes containing 20 μL of BP extract to be tested. After incubation for 2 h at 37 °C with shaking, β-gal and AP were assayed. The induction factor (IF) was calculated as the ratio of R_c/R_o, where R_c is equal to β-gal activity/AP activity determined at concentration c of the tested extract and R_o is equal to β-gal activity/AP activity in the absence of the tested extract. A substance is defined as genotoxic when IF > 2.

The same procedure was used also in the presence of a human liver S9 pool (containing 3.34 nmol/mL cytochrome P450 and 27 mg/mL proteins; Molecular Toxicology, Inc., Moltox, Boone, NC) used as an exogenous metabolic activation system. The S9 mixture (0.4 cc/tube) was activated according to Quillardet and Hofnung (*10*). To monitor the efficiency of the S9 activation system under our experimental conditions, the formation of a single active metabolite from piperacilline (20 μg/assay) was monitored by high-performance liquid chromatography (HPLC).

Each BP extract was dissolved in dimethyl sulfoxide (DMSO) and tested in triplicate. Their genotoxic activity was evaluated in comparison to that of the indirect-acting mutagen benzo[*a*]pyrene (B[*a*]P; 2.5 μg/assay) and the direct-acting mutagen 4-nitroquinoline-*N*-oxide (4-NQO; 0.02 μg/assay), used as positive internal controls.

Effect of HUVECs Exposed to TNF-α. *Cell Culture and Treatment*. HUVECs, isolated from freshly obtained human umbilical cords by collagenase digestion of the interior of the umbilical vein (*11*), were cultured in medium 199 supplemented with 20% fetal bovine serum, L-glutamine, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), penicillin/streptomycin, and endothelial cell growth factor (50 mg/mL)

in gelatin-pretreated flasks and maintained at 37 °C in a humid 5% CO_2 atmosphere incubator. HUVECs were used within the passages 2−4. For all experiments, the extracts 100 E1 and 70 E2 were dissolved in DMSO and used immediately. The final concentration of DMSO in culture medium was 0.1% (v/v). The subconfluent cells were incubated with the extracts (50−75−100 μg/mL) for 24 h at 37 °C; control cells were treated with their vehicle only. Then, the cells were washed with phosphate-buffered saline and incubated for 2 h in the presence of 20 ng/mL TNF-α or its vehicle (distilled water). The amount of proteins in the cell lysates was detected by the Bradford assay (*12*).

Cell Viability. Cell viability was evaluated by the trypan blue exclusion assay.

Glutathione Levels. The intracellular concentration of GSH and GSSG was detected by HPLC (Shimadzu, Milan, Italy) equipped with a fluorescence detector (Hewlett-Packard 1046A) (*13*). Briefly, cells were lysed and centrifuged, and the supernatant was used to determine the content of total glutathione (GSH + GSSG) and GSH by HPLC analysis of the GSH-*o*-phthalaldehyde (OPA) adducts. To determine GSH, the proteins in the supernatant were precipitated with 2.5% 5-sulfosalicylic acid (SSA, w/v) and centrifuged; then, the deproteinized supernatant was used directly for OPA derivatization. To determine total glutathione, 100 μL of cell lysate was mixed with 100 μL of 25 mM dithiothreitol and 50 μL of 0.1 M Tris at pH 8.5 and kept on ice for 30 min to allow for disulfide reduction. Then, proteins were precipitated with 2.5% SSA (w/v), and after centrifugation, the supernatant was used for OPA derivatization. Derivatization with OPA was performed at room temperature by mixing 200 μL of the deproteinized supernatant with 200 μL of 5 mg/mL OPA. After 1 min, the samples were neutralized and diluted by the addition of 2 mL of 100 mM sodium phosphate at pH 7.0.

Chromatographic separation was carried out by means of a C18 reverse-phase column (Phenomenex) maintained at 25 °C. The mobile phase was 0.15 M sodium acetate at pH 7.0 containing 7.5% (v/v) methanol; the flow rate was 1 mL/min. The fluorescence detector was set at λ_{ex} = 340 nm and λ_{em} = 420 nm. An external standard calibration method was used for GSH quantification by serial dilutions of a GSH solution undergoing OPA derivatization.

SOD Activity. SOD activity in cell lysate was assayed by a spectrophotometric test (*14*) based on the reduction of nitro blue tetrazolium (NBT; maximum absorbance at 560 nm) by the superoxide radical anion generated by a β-nicotinamide adenine dinucleotide/phenazine methosulfate system. Results were expressed as SOD units/mg of protein. One SOD unit is taken as the amount of enzyme required to give 50% inhibition of NBT reduction.

MDA/HNE. A chromogenic reagent, *N*-methyl-2-phenylindole, dissolved in acetonitrile, reacting with MDA and HNE at 45 °C was used. Condensation of 1 molecule of either MDA or HNE with 2 molecules of reagent yields a stable chromophore with maximal absorbance at 586 nm.

NF-κB Activation Status by the Electrophoretic Mobility Shift Assay (EMSA). The cell pellet was resuspended in 400 μL of ice cold buffer A (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 5% glycerol, and protease inhibitors). After 15 min of incubation on ice, cells were lysed by adding 25 μL of 10% Nonidet P40. Nuclei were recovered by centrifugation and lysed in 50 μL of ice cold buffer B [20 mM HEPES at pH 7.9, 400 mM NaCl, 1 mM $MgCl_2$, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, and protease inhibitors]. Sample protein concentrations were brought to 5 μg/μL with buffer B. Double-stranded oligonucleotides (Life Technologies) were prepared, annealing for 10 min 1 nmol of each complementary oligonucleotide in 1× PCR buffer (Promega) by heating at 95 °C for 10 min. A total of 10 pmol of annealed oligonucleotides was labeled in 1× kinase buffer with 10 units of T4 polynucleotide kinase (Amersham) and 10 μCi [γ^{32}P]-ATP (NEN) at 37 °C for 40 min. After kinase inactivation, duplex oligonucleotides were purified using Micro Biospin30 columns (Biorad). Binding reactions were performed incubating 5 μg of nuclear proteins for 20 min at room temperature with 2 μg of poly(dI-dC)·poly(dI-dC) (Amersham) and 50 000 cpm (Cherenkov counting) of labeled oligonucleotides. DNA protein complexes were resolved in 6% polyacrylamide (29:1 acrylamide/bisacrylamide) gels and then autoradiographed. The specificity of the DNA binding was also confirmed using binding reactions containing 100-fold excess of unlabeled oligonucleotides as cold competitors (not shown). Oligonucleotide consensus sequences for NF-κB are from 5′ to 3′: AGT TGA GGG GAC TTT CCC AGG C.

Table 2. Total Phenol Content and DPPH Radical-Scavenging Activity of the BP Extracts 70 E2 and 100 E1[a]

sample	total phenols (μg/mg)	DPPH test (μg/mL) SC_{50} (CL 95%)
70 E2	48.97 ± 2.20	254.42 (160.25−320.85)
100 E1	40.93 ± 2.02	481.25 (358.25−602.56)
Trolox	189.83 ± 5.20	25.46 (20.09−32.28)

[a] Total phenols are expressed as micrograms of gallic acid equivalents per milligram of extract; values represent the mean ± SD of three independent experiments. Scavenging concentrations (SC_{50}) are expressed as μg/mL of extract.

Release of Prostaglandin E_2 (PGE_2) and Thromboxane B_2 (TxB_2) in Whole Blood. Peripheral blood samples were drawn from healthy volunteers who had taken no nonsteroidal anti-inflammatory drugs (NSAIDs) or steroid drugs during the last 2 weeks before the study. To evaluate changes in PGE_2 release, 1 mL heparinized peripheral blood aliquots were incubated in the presence of lypopolysaccharide (LPS; 10 μg/mL) for 24 h at 37 °C (*15*). The contribution of platelet COX-1 was suppressed by adding aspirin (10 μg/mL) at time 0. Then, plasma was separated by centrifugation and kept at −30 °C until assayed for the content of PGE_2, which is an index of blood monocyte COX-2 activity. To evaluate changes in TxB_2 release, 1 mL whole blood aliquots were immediately transferred into glass tubes and allowed to clot at 37 °C for 1 h. Serum was separated by centrifugation and kept at −30 °C until assayed for TxB_2, which is an index of endogenously formed thrombin-stimulated platelet COX-1 activity. Plasma PGE_2 and serum TxB_2 were measured by the enzyme immunoassay (EIA).

To evaluate their effects on PGE_2 and TxB_2 release in whole blood, the BP extracts (dissolved in DMSO, 2 μL/tube) were added to whole blood to give a 200−600 μg/mL final concentration. The experiments were then carried out as described above.

Statistical Analysis. Results are expressed as the mean ± standard deviation (SD) of three experiments and statistically analyzed by the analysis of variation (ANOVA) test. Differences among groups and treatments were considered significant for $p < 0.05$.

RESULTS AND DISCUSSION

Consumption of plant-derived foods and beverages rich in polyphenols may represent a beneficial diet in terms of vascular protection, and dietary supplements containing polyphenols and antioxidants can improve endothelial function. Recently, particular attention has been given to flavonoid-rich phytocomplexes from *Citrus*, as anti-inflammatory agents potentially active also at the level of the endothelial system. This study was carried out with the aim to evaluate the protective effect of two BP extracts rich in flavonoids on human endothelial cells exposed to TNF-α.

The radical scavenger properties of these extracts have been preliminary examined in the DPPH assay and the Folin−Cioacalteau test. The radical-scavenging activities of BP extracts, measured as decolorizing activity following the trapping of the unpaired electron of DPPH, are shown in **Table 2**. Both of the extracts showed good DPPH antiradical activities, with IC_{50} values of 254.42 μg/mL for 70 E2 and 481.25 μg/mL for 100 E1. These findings are in agreement with those observed in the Folin−Ciocalteau assay. In fact, the total phenol content for 70 E2 and 100 E1 are 48.97 and 40.93 μg/mg of extract, respectively.

However, accumulating data from *in vitro* and *in vivo* studies continue to show that some antioxidant phytochemicals could possess genotoxic and/or mutagenic effects by themselves or could potentiate the effect of other xenobiotics. The SOS chromotest assay, a short-term bacterial assay useful to give an estimation of the genotoxic potential of substances (*10*, *18*), was employed in our study to determine the genotoxic effect of the BP

extracts, in both presence or absence of an exogenous metabolic activation system S9. Our findings show that the two BP extracts, used at doses up to 50 μg/assay, exhibit no genotoxic effect, also if undergoing enzymatic metabolization (**Tables 3** and **4**). This demonstrates that the BP extracts tested do not produce DNA lesions blocking DNA synthesis and leading to SOS system induction. The lacking of genotoxic effects because of bioactivation of the compounds contained in these BP extracts was confirmed by HPLC analyses, which showed identical chemical profiles for the extracts before and following exposure to the S9 mix (data not shown).

Table 3. Genotoxic Effect of the BP Extracts 100 E1 and 70 E2 in the Absence of the Exogenous Metabolizing System S9 in the SOS Chromotest[a]

		E. coli PQ37		
sample	μg/assay	β-galactosidase (units)	alkaline phosphatase (units)	IF
4-NQO	0.02	15.88 ± 0.002	8.38 ± 0.001	7.78
100 E1	0	2.13 ± 0.001	8.73 ± 0	1
	6.25	2.15 ± 0.002	7.63 ± 0.001	1.16
	12.50	2.45 ± 0	7.80 ± 0.005	1.29
	25.00	2.53 ± 0.001	7.88 ± 0	1.32
	50.00	2.68 ± 0.002	7.75 ± 0.003	1.42
	100.00	2.83 ± 0.001	7.95 ± 0.002	1.46
70 E2	0	2.18 ± 0.001	8.73 ± 0	1.00
	6.25	2.08 ± 0.002	7.58 ± 0.001	1.10
	12.50	2.25 ± 0.005	7.63 ± 0.002	1.18
	25.00	2.68 ± 0.001	7.75 ± 0	1.38
	50.00	2.95 ± 0	7.63 ± 0	1.55
	100.00	3.03 ± 0.01	7.55 ± 0.02	1.61

[a] Data are expressed as the mean ± SD of three independent experiments. 4-NQO was used as a positive control. IF = inhibition factor.

Table 4. Genotoxic Effect of the BP Extracts 100 E1 and 70 E2 in the Presence of the Exogenous Metabolizing System S9 in the SOS Chromotest[a]

		E. coli PQ37		
sample	μg/assay	β-galactosidase (units)	alkaline phosphatase (units)	IF
B[*a*]P	2.5	12.00 ± 0.003	8.88 ± 0.001	5.18
4-NQO	0.02	15.88 ± 0.002	8.43 ± 0.001	7.56
100 E1	0	2.38 ± 0.002	8.63 ± 0	1
	6.25	2.48 ± 0.001	7.63 ± 0.001	1.18
	12.50	2.63 ± 0.003	7.38 ± 0.004	1.29
	25.00	2.88 ± 0.001	7.63 ± 0	1.37
	50.00	3.38 ± 0.001	8.33 ± 0.003	1.47
	100.00	3.63 ± 0.00	8.48 ± 0.001	1.55
70 E2	0	2.25 ± 0.001	8.63 ± 0.002	1.00
	6.25	2.30 ± 0.002	8.08 ± 0.003	1.09
	12.50	2.53 ± 0.003	8.00 ± 0.002	1.21
	25.00	2.88 ± 0.001	7.80 ± 0.002	1.41
	50.00	3.15 ± 0	7.88 ± 0	1.53
	100.00	3.35 ± 0.001	8.13 ± 0.002	1.58

[a] Data are expressed as the mean ± SD of three independent experiments. B[*a*]P and 4-NQO were used as positive controls. IF = inhibition factor.

The potential protective effect of BP extracts against endothelial dysfunction was investigated by testing their capability to alter the response to TNF-α in HUVECs, which are a model system widely used to identify the effects of and targets for deleterious vascular risk factors, such as oxidative stress on endothelial cells. TNF-α was selected as a stressor because it has been shown that TNF-α-induced cell responses, particularly in endothelial cells, are mediated through its ability to promote intracellular ROS formation.

In our experiments, HUVECs were exposed for 2 h to 20 ng/mL TNF-α. Under these experimental conditions, TNF-α induced a significant decrease in cell viability, with almost 35% cell death (**Figure 1**), as reported elsewhere (*2*). As reported in the literature and also preliminarily confirmed in our laboratories, this level of HUVEC exposure to TNF-α can induce significant necrotic cell death, without evidence of apoptotic activation.

TNF-α is known to dose-dependently increase intracellular MDA and 4-HNE (lipid peroxidative metabolites resulting from the interaction of ROS and free radicals with lipids and, thus, indirect indicators of oxidative stress) in HUVECs (*16*), and the ratio GSH/GSSG, which thus reflects the redox status within the cells, has been demonstrated to be reduced in HUVECs exposed to TNF-α (*17*).

Thus, we monitored, as hallmarks of oxidative damage in TNF-α-exposed HUVECs, the intracellular levels of MDA/HNE, GSH and GSSG, and SOD activity. Exposure of HUVECs to TNF-α increased lipid peroxidation byproduct levels (MDA and HNE) with respect to unexposed control cells (**Figure 2**). Furthermore, we confirmed that TNF-α exposure altered the levels of endogenous antioxidant defense enzymes (**Table 4** and **Figure 3**). In fact, TNF-α-exposed HUVECs presented increased GSSG levels, with a consequent reduction in the GSH/GSSG ratio (**Table 5**). Moreover, the levels of SOD activity in TNF-α-challenged HUVECs were significantly lower than those measured in control cells.

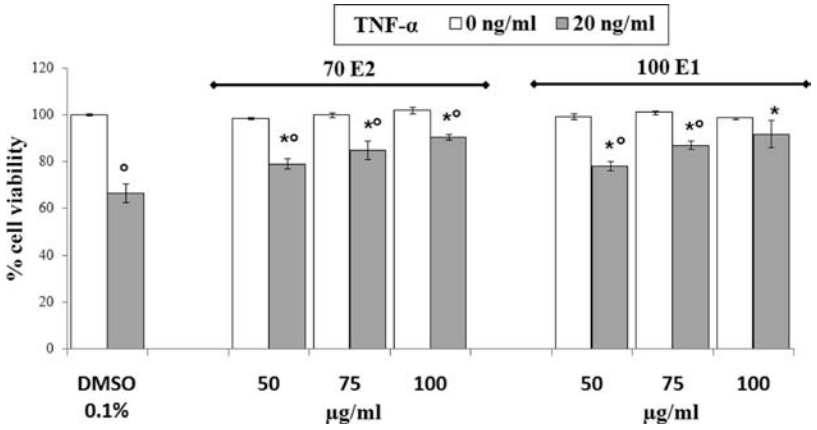

Figure 1. Viability of HUVECs pretreated for 24 h with the BP extracts 70 E2 and 100 E1 (50−75−100 μg/mL) or their vehicle (0.1% DMSO) and then exposed for 2 h to TNF-α (20 ng/mL) or its vehicle (distilled water; 0 ng/mL TNF-α). Data represent the percentage of viable cells (mean percentage) in treated samples with respect to the control (0.1% DMSO + 0 ng/mL TNF-α). Each point is the mean ± SD of three independent experiments. (○) $p < 0.01$ versus the respective 0 ng/mL TNF-α. (∗) $p < 0.01$ versus 0.1% DMSO exposed to 20 ng/mL TNF-α.

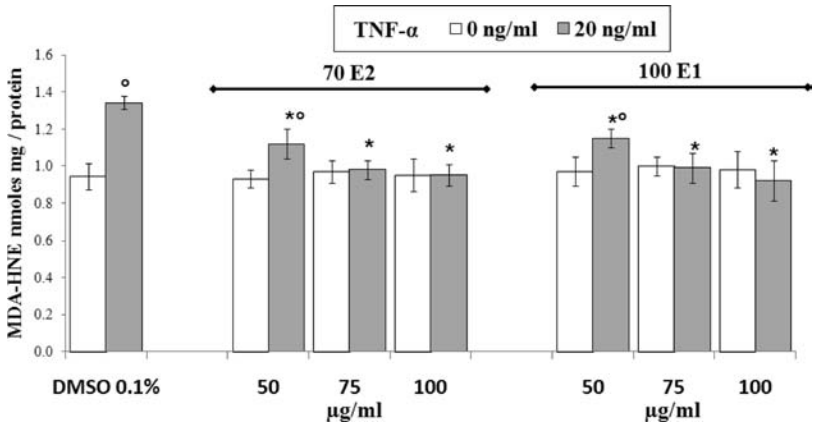

Figure 2. Levels of MDA and 4-HNE in HUVECs pretreated for 24 h with the BP extracts 70 E2 and 100 E1 (50−75−100 μg/mL) or their vehicle (0.1% DMSO) and then exposed for 2 h to TNF-α (20 ng/mL) or its vehicle (distilled water; 0 ng/mL TNF-α). Each point is the mean ± SD of three independent experiments. (○) $p < 0.01$ versus the respective 0 ng/mL TNF-α. (∗) $p < 0.01$ versus 0.1% DMSO exposed to 20 ng/mL TNF-α.

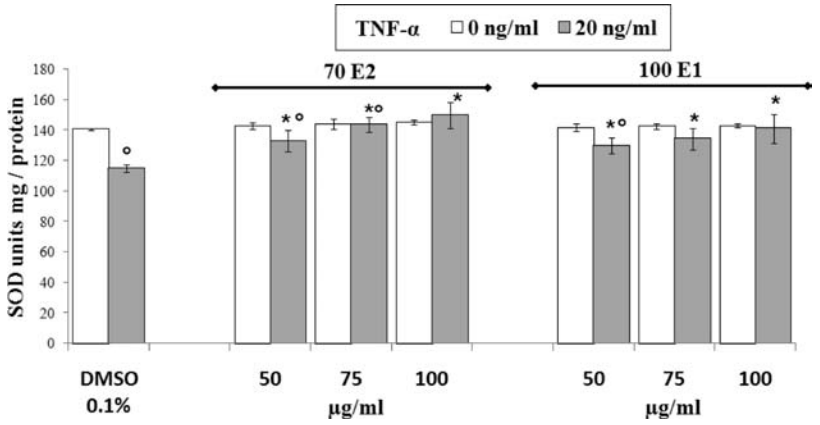

Figure 3. SOD activity of HUVECs pretreated for 24 h with the BP extracts 70 E2 and 100 E1 (50−75−100 μg/mL) or their vehicle (0.1% DMSO) and then exposed for 2 h to TNF-α (20 ng/mL) or its vehicle (distilled water; 0 ng/mL TNF-α). Each point represents the mean ± SD of three independent experiments. (○) $p < 0.01$ versus the respective 0 ng/mL TNF-α. (∗) $p < 0.01$ versus 0.1% DMSO exposed to 20 ng/mL TNF-α.

Table 5. Changes in GSH and GSSG Levels and GSH/GSSG Ratio in HUVECs Pretreated for 24 h with the BP Extracts 70 E2 and 100 E1 (50−75−100 μg/mL) or Their Vehicle (0.1% DMSO) and Then Exposed for 2 h to TNF-α (20 ng/mL) or Its Vehicle (Distilled Water)[a]

	GSH (nmol/mg of protein)	GSSG (nmol/mg of protein)	ratio of GSH/GSSG
0.1% DMSO + 0 ng/mL TNF-α	36.3 ± 0.7	5.2 ± 0.3	7.0 ± 0.4
50 μg/mL 70 E2 + 0 ng/mL TNF-α	37.0 ± 0.5	5.1 ± 0.5	7.3 ± 0.6
75 μg/mL 70 E2 + 0 ng/mL TNF-α	37.3 ± 0.6	5.1 ± 0.6	7.3 ± 0.7
100 μg/mL 70 E2 + 0 ng/mL TNF-α	37.9 ± 0.7	5.0 ± 0.7	7.5 ± 0.7
50 μg/mL 100 E1 + 0 ng/mL TNF-α	36.8 ± 0.6	5.0 ± 0.4	7.4 ± 0.4
75 μg/mL 100 E1 + 0 ng/mL TNF-α	37.1 ± 0.7	4.9 ± 0.6	7.6 ± 0.7
100 μg/mL 100 E1 + 0 ng/mL TNF-α	37.5 ± 0.7	4.9 ± 0.9	7.6 ± 0.7
0.1% DMSO + 20 ng/mL TNF-α	34.3 ± 0.1°	12.5 ± 1.2°	2.9 ± 0.9°
50 μg/mL 70 E2 + 20 ng/mL TNF-α	35.1 ± 1.3	8.5 ± 0.6*°	4.1 ± 0.7°
75 μg/mL 70 E2 + 20 ng/mL TNF-α	36.0 ± 1.0*	6.8 ± 0.2*°	5.3 ± 0.1*°
100 μg/mL 70 E2 + 20 ng/mL TNF-α	36.4 ± 0.3*°	4.9 ± 0.2*	7.4 ± 0.2*
50 μg/mL 100 E1 + 20 ng/mL TNF-α	34.5 ± 0.8°	8.8 ± 0.5*°	3.9 ± 0.5°
75 μg/mL 100 E1 + 20 ng/mL TNF-α	34.8 ± 1.2°	7.3 ± 0.7*°	4.8 ± 0.8°
100 μg/mL 100 E1 + 20 ng/mL TNF-α	34.9 ± 1.2°	5.3 ± 0.4*	6.6 ± 0.4*

[a] Each point represents the mean ± SD of three independent experiments. (○) $p < 0.01$ versus the respective 0 ng/mL TNF-α. (∗) $p < 0.01$ versus 0.1% DMSO exposed to 20 ng/mL TNF-α.

The pretreatment for 24 h with both the BP extracts at concentrations up to 100 μg/mL appeared unable to affect viability of control cells not exposed to TNF-α, as well as intracellular levels of MDA/HNE, GSH/GSSG, and SOD activity.

These extracts showed an evident protective effect against the alterations following 2 h exposure to 20 ng/mL TNF-α (**Figure 1**). In fact, after pretreatment for 24 h with 100 E1 and 70 E2, a dose-dependent increase of cell survival was observed for cells exposed to TNF-α. These data clearly indicate that pretreatment of vessel endothelial cells with bergamot extracts can significantly inhibit TNF-α-induced cell death.

Cell pretreatment of HUVECs with 100 E1 and 70 E2 (50−75−100 μg/mL) proved also to prevent the oxidative stress-related damage induced by TNF-α exposure. In fact, no increase

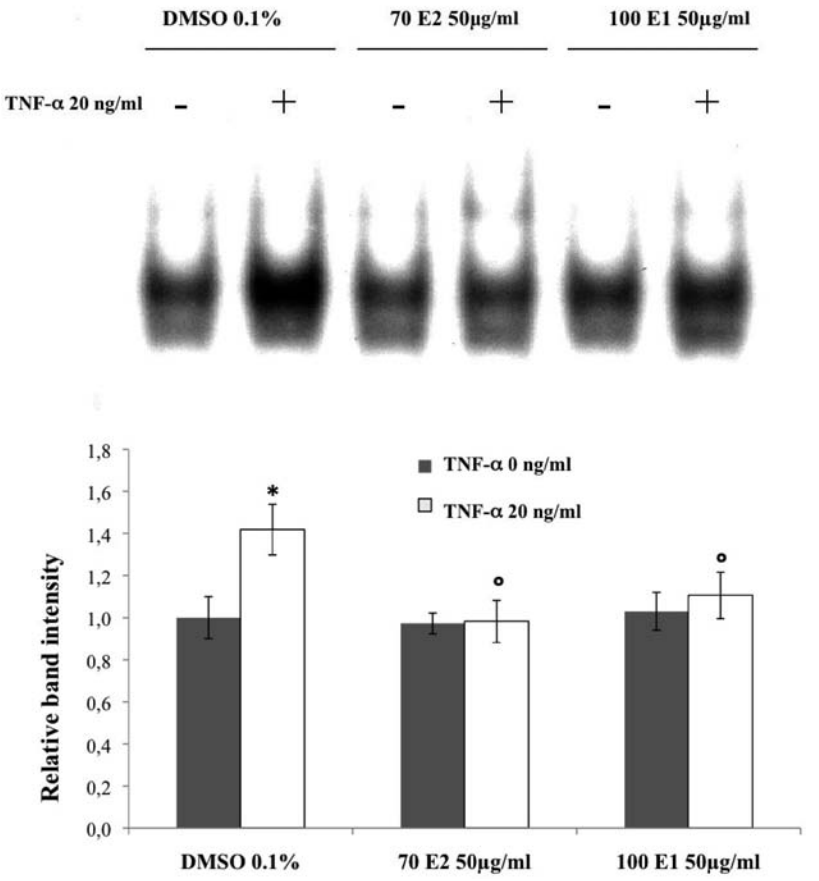

Figure 4. Modulation of NF-κB activation in HUVECs by BP extract pretreatment. Cells pretreated for 24 h with the BP extracts 70 E2 and 100 E1 (50 μg/mL) or their vehicle (0.1% DMSO) and then exposed for 2 h to TNF-α (20 ng/mL) or its vehicle (distilled water; 0 ng/mL TNF-α). Nuclear extraction was performed 2 h after TNF-α exposure. The specificity of the DNA binding was confirmed using binding reactions containing 100-fold excess of unlabeled oligonucleotides as cold competitors (not shown). Results, from at least three separate experiments, are indicated as the mean ± SD of the relative densitometric intensity and expressed as a fold of increase versus untreated and unexposed cells. (∗) $p < 0.01$ versus 0.1% DMSO unexposed to TNF-α. (○) $p < 0.01$ versus 0.1% DMSO exposed to TNF-α.

in intracellular MDA/HNE levels was observed when TNF-α-challenged HUVECs were pretreated with the BP extracts. Cell pretreatment with 100 E1 and 70 E2 was also able to restore cellular SOD activity in a dose-dependent way. Accordingly, with these findings, pretreatment of HUVECs with the BP extracts completely prevented TNF-α-induced changes in GSSG levels and the GSH/GSSG ratio.

Cell signaling pathways converging on redox-sensitive transcription factors, such as NF-κB, are involved in mediating inflammatory response. NF-κB activation has been linked to a wide range of diseases, and in particular, uncontrolled or chronic NF-κB activation is a hallmark of chronic inflammatory diseases. In our study, we used the activation status of NF-κB to assess HUVEC responses to oxidants. No change in NF-κB signaling was observed when TNF-α unexposed cells were treated to BP extracts, at all doses used (50, 75, and 100 μg/mL; data not shown). However, also at the low dosage level used in our study (50 μg/mL), both of the BP extracts after a 24 h incubation period showed a significant pattern of decreased TNF-α-stimulated NF-κB signaling (**Figure 4**). Thus, the reductions in NF-κB signaling appear to be a direct result of the activity of the BP extracts on the NF-κB system probed by TNF-α.

Our results represent clear evidence that the BP extracts can protect human vessel endothelium from TNF-α-induced damage by preventing the alterations in the intracellular redox status caused by this inflammatory cytokine. This effect is very likely due to the significant radical scavenger properties of polyphenols (particularly flavonoids) contained in them.

One has to note that, in our experiments, the two BP extracts, despite a different flavonoid content (**Table 1**), showed similar protective power on TNF-α-challenged HUVECs. The 70 E2 extract and, at a very low degree, the 100 E1 extract contain discrete amounts of glucose and small amounts of other monosaccharides; furthermore, because of the composition of the BP, BP extracts can also contain trace amounts of pectins (*6*). Finally, on the basis of the antimicrobial properties of these extracts, both 70 E2 and 100 E1 seem to contain, besides flavonoids, other active unidentified phytochemicals (*8*). All of these components can influence the antioxidant and anti-inflammatory activity of the BP extracts both directly (because of their own properties) and indirectly (acting as a synergist or antagonist, as well as influencing the access of other active principles to the target). Thus, this complex composition can explain why the extract 70 E2, although its flavonoid content is significantly higher than that of the 100 E1, showed antioxidant and endothelial protective capability similar to that of 100 E1.

Our findings are also in agreement with several papers showing *in vitro* and *in vivo* protective effects of plant polyphenols against TNF-α-induced endothelial activation and dysfunction (*19*). Recently, Miyake and others (*20*) demonstrated that flavonoids from lemon juice are endowed with antioxidant properties and can reduce ICAM-1 expression induced by TNF-α in HUVECs (*20*).

Furthermore, Kamata and co-workers reported that *Citrus unshiu* MARC extract preserves aorta endothelial function in STZ-induced diabetic rats without lowering plasma cholesterol, because of the radical scavenger/antioxidant activities of its active components (*21*).

Although, the mechanisms underlying the beneficial effects of plant polyphenols on vessel endothelium are not clearly understood, many authors suggest that these phytochemicals act through an interaction with the NF-κB pathway (*19*). In fact, a wide variety of plant-derived agents can alter or correct undesired cellular functions caused by abnormal pro-inflammatory signal transmission mediated by inappropriately activated NF-κB (*22*). Our present findings demonstrate that the phytochemicals present in the BP extracts can downregulate the NF-κB signal transduction pathway, which responds to oxidative signals.

The flavonoid apigenin has been shown to significantly inhibit the inflammation induced by TNF-α in HUVECs, by suppressing the upregulation of adhesion molecules. However, its anti-inflammatory properties might also be related to the capability to inhibit COX-2 expression, as demonstrated in LPS-exposed macrophages (*23*). COX-1 is generally expressed constitutively; its expression may be, however, regulated by certain cytokines. Conversely, COX-2 is rarely expressed constitutively, but it is highly induced by cytokines, growth factors, and tumor promoters. TNF-α induces COX-2 expression in HUVECs through a mitogen-activated protein kinase (MAPK) p38-dependent pathway, in which a main step is represented by intracellular ROS generation and GSH/GSSG ratio reduction (*17*). However, we could exclude that the protective effect of BP extracts against TNF-α-induced changes in HUVECs may be due to their capability to inhibit COX-1 or COX-2 pathways because these phytocomplexes, when tested on human whole blood at doses up to 100 μg/assay, appeared unable to modify PGE_2 and TXB_2 release.

In conclusion, the present study has demonstrated that BP, a byproduct of the *Citrus* fruit processing and essential oil industries, is a potential source of natural antioxidant/anti-inflammatory compounds. BP is still rich in sugars, fibers, and other residual substances and, therefore, causes economic and environmental problems because of its fermentability. These findings provide evidence of a clear scope to process BP not only as an animal feed but also as a potential source of *Citrus* phytocomplexes to be used in nutraceutical products or functional foods. A further advantage of using BP waste is the very low content of psoralens, which may act as photosensitizers and initiate procarcinogenic events. Finally, these flavonoid-enriched extracts are quickly and easily generated from the original peel waste using processes and solvents that have a negligible impact on the environment.

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