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ORIGINAL CONTRIBUTION

Polymeric proanthocyanidins from Sicilian pistachio (*Pistacia vera* L.) nut extract inhibit lipopolysaccharide-induced inflammatory response in RAW 264.7 cells

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Abstract
Background Positive effects of pistachio nut consumption on plasma inflammatory biomarkers have been described; however, little is known about molecular events associated with these effects.
Purpose We studied the anti-inflammatory activity of a hydrophilic extract from Sicilian *Pistacia* L. (HPE) in a macrophage model and investigated bioactive components relevant to the observed effects.
Methods HPE oligomer/polymer proanthocyanidin fractions were isolated by adsorbance chromatography, and components quantified as anthocyanidins after acidic hydrolysis. Isoflavones were measured by gradient elution HPLC analysis. RAW 264.7 murine macrophages were pre-incubated with either HPE (1- to 20-mg fresh nut equivalents) or its isolated components for 1 h, then washed before stimulating with lipopolysaccharide (LPS) for 24 h. Cell viability and parameters associated with Nuclear Factor-κB (NF-κB) activation were assayed according to established methods including ELISA, Western blot, or cytofluorimetric analysis.
Results HPE suppressed nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production and inducible NO-synthase levels dose dependently, whereas inhibited prostaglandin E_2 (PGE_2) release and decreased cyclo-oxygenase-2 content, the lower the HPE amount the higher the effect. Cytotoxic effects were not observed. HPE also caused a dose-dependent decrease in intracellular reactive oxygen species and interfered with the NF-κB activation. Polymeric proanthocyanidins, but not isoflavones, at a concentration comparable with their content in HPE, inhibited NO, PGE_2, and TNF-α formation, as well as activation of IκB-α. Oligomeric proanthocyanidins showed only minor effects.
Conclusions Our results provide molecular evidence of anti-inflammatory activity of pistachio nut and indicate polymeric proanthocyanidins as the bioactive components. The mechanism may involve the redox-sensitive transcription factor NF-κB. Potential effects associated with pistachio nut consumption are discussed in terms of the proanthocyanidin bioavailability.

Keywords Inflammation · Isoflavones · Macrophages · Nut · Proanthocyanidins · Sicilian pistachio

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Introduction

Characterizing foods and food components that may affect specific cellular events involved in body's pathophysiologic processes and reduce the risk of chronic diseases is a main target of the modern nutritional science. The beneficial effects on health of nuts and seeds, whole foods rich of unsaturated fatty acids, plant proteins, dietary fiber, antioxidant vitamins, phenolic compounds, and salutary minerals [1–4], have been investigated for a number of years. In particular, it is currently acknowledged that nut consumption lowers cardiovascular disease (CVD) risk, which has been related with the ability to improve conventional risk factors, including serum concentrations of total and low-density lipoprotein cholesterol and platelet aggregation [5–11]. Pistachio (*Pistacia* genus) is a member of the Anacardiaceae family native of the Mediterranean basin and of arid zones of Asia. Essential oils and lipophilic extracts of various parts of the plant including leaf, branch, stem, and seeds have been studied in vitro and in vivo for various healthy activities, with terpenoid derivatives playing a major role in the observed effects [12–15]. Among *Pistacia* species, *P. vera* L. is grown for its edible nuts. Studies on properties and bioactivity of this nut to date are limited with respect to other nuts such as walnuts, peanuts, and almonds. Pistachio nut consumption has been shown to have positive effects on serum lipid profile and CVD risk factors in healthy as well as hypercholesterolemic humans [16–19]. Potential mechanisms of the cardiovascular protective action have been suggested, including effects on plasma stearoyl-CoA desaturase activity [19]. More recent studies in healthy subjects showing that a pistachio diet for 4 weeks significantly improves endothelial function and oxidative status and is associated with lower levels of circulating inflammatory biomarkers [20] may suggest that anti-inflammatory activities of nut components play a role in preventing pathogenetic events leading to CVD. Systemic markers of inflammation have been demonstrated strong predictors of cardiovascular disease [21–23]. Anti-inflammatory effects of pistachio nut and anti-inflammatory activity of its components have not been explored at a molecular level yet.

Inflammation is a multifactorial process occurring in several sequential steps at the level of which oxidative stress and reactive oxygen species may have different roles. Indeed, they may be involved in pathogenesis and have regulatory activity as well as play a key role in enhancing the inflammatory response thus favoring starting of chronic inflammatory states. Macrophages are effector cells in inflammatory reactions. Control of the redox status of these cells is essential to modulate signal transduction pathways involved in the production and/or activation of a number of pro-inflammatory mediators, including cytokines, transcription factors, enzymes, and reactive oxygen and nitrogen species (ROS), as well as to maintain their own vital function. We have recently shown that a hydrophilic extract from Sicilian *P. vera* fresh nuts (HPE) contains substantial amounts of bioactive polyphenols [24]. In the present study, the activity of HPE and of its polyphenol components has been explored in vitro in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages, a well-established cell model to investigate molecular pathways of the inflammatory response, in the attempt to rationalize anti-inflammatory effects of pistachio nut diets.

Materials and methods

Reagents

LPS from Escherichia coli 0127:E8, benzamidine, cyanidin chloride, (+) cathechin, 2′,7′-dichlorofluorescin diacetate (DCFDA), daidzein, dimethyl-sulphoxide (DMSO), dithiothreitol (DTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), genistein, methylenediamine tetraacetic acid (EDTA), HEPES buffer solution, pepstatin A, and phenyl-methanesulphonylfluoride (PMSF) were from Sigma Chemical Co (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and gentamicin were from Gibco-BRL (Gaithersburg, MD, USA). Monoclonal antibodies against iNOS, COX-2, p65, phospho-IκB-α, β-actin, and poly (ADP-ribose)polymerase (PARP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-IgG antibody was from DAKO (Glostrup, Denmark).

Preparation of hydrophilic pistachio extract (HPE)

Pistacia vera L. nuts, Bronte's cultivar, were shelled, and the kernels with their skin were powdered in a mortar. Samples (25 g) were then extracted with 200 mL of a mixture of methanol/water (2:1; v/v) over 24 h at 4 °C. After a clean-up step via centrifugation and filtration through a Millex HV 0.45-μm filter (Millipore, Billerica, MA), the organic solvent was evaporated in a rotary vacuum evaporator, and the aqueous solution submitted to cryo-dessiccation, stored at −80 °C, and used within 6 months. The freeze-dried samples were tested on LPS-activated RAW 264.7 macrophages and/or purified for further studies.

Proanthocyanidin fractionation

Oligomer/polymer fractionation from HPE was carried out following Jordao et al. [25]. Briefly, cryo-dessicated HPE was re-suspended with water to reach 10 g/mL. Aliquots were passed through two neutral Sep-Pack cartridges connected in series and preconditioned with 10 mL water adjusted to pH 7.0. After elution with 4 mL water (pH 7.0), the column was dried with N_2. Flavonoids (isoflavones), and oligomeric proanthocyanidins were first eluted with 25 mL ethyl acetate, and then, the polymeric fraction (PF) was collected with 10 mL methanol. The ethyl acetate fraction was evaporated to dryness under vacuum at 25 °C, dissolved in distilled water, and then re-deposited onto the same connected cartridges preconditioned with distilled water. After drying the cartridges with N_2, the cartridge was eluted sequentially with 25 mL diethyl ether and finally with 10 mL methanol (oligomeric fraction, OF). The organic solvent of PF and OF was evaporated in a rotary vacuum evaporator.

HPE, OF, and PF were re-suspended in suitable volumes of 5 mM phosphate-buffered saline, pH 7.4 (PBS), and proanthocyanidins were quantified according to the modified method of Porter et al. [26], after conversion to anthocyanidins by acidic hydrolysis in the presence of iron ions. Briefly, 0.5 mL of HPE, OF, or PF was added to 1.5 mL of EtOH/HCl (95:5; v/v) and 50 μL of 2 mM $FeCl_3$ dissolved in 2 M HCl. The reaction mixture was capped and heated in a water bath at 95 °C, for 40 min. After cooling in cold water, the anthocyanidins formed were evaluated spectrophotometrically by measuring the peak height at 543 nm over a baseline between 400 and 700 nm. The amount of proanthocyanidins in HPE was corrected for the contribution from anthocyanins as described [24]. Proanthocyanidin concentration was expressed as the amount of cyanidin formed according to a calibration curve with cyanidin chloride.

Isoflavone analysis

HPLC evaluation of phytoestrogenic isoflavones daidzein and genistein in HPE was performed with a Gilson modular liquid chromatographic system (Gilson Inc., Middleton, WI), on a RP-18e Performance column (100 × 4.6 mm; Merck, Darmstadt, Germany), equipped with RP-18e Chromolith guard cartridge (5 × 4.6 mm, Merck) using a gradient elution. Solvent A was 0.1% (v/v) acetic acid in water, solvent B was 0.1% (v/v) acetic acid in acetonitrile, and the flow-rate was 1 mL/min. The gradient was started immediately upon injection, and gradient elution was from 10 to 70% B in a linear gradient over 60 min. The column was washed at 90% B for 3 min and equilibrated 10 min between runs at 10% B. Detection was at 260 nm. The isoflavones were quantified by reference to standard curves constructed with 1 to 100 ng of each pure commercial compound.

Cell culture

The RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (Cryosite, Lane Cove NSW, Australia). Cells were grown in DMEM supplemented with 10% FBS and 5 μg/mL gentamicin in humidified 5% CO_2 atmosphere, at 37 °C. In all experiments, RAW 264.7 cells were seeded in triplicate in 24-well culture plates at a density of 2.5×10^5 cells/well and allowed to adhere for 2 h. Unless specified, the cells were pre-treated with either HPE or proanthocyanidin fractions re-suspended in DMEM, or isoflavones re-suspended in DMSO, for 60 min. The cells were washed, and then, the medium was replaced with fresh DMEM, and cells stimulated with 1 μg/mL LPS for a suitable time-length. DMSO never exceeded 0.1%. Cells pre-treated with vehicle alone were taken as control in all experiments.

MTT assay for cell viability

RAW 264.7 cells were plated at 5×10^4 cells/well in 96-well plates containing 200 μL DMEM and allowed to adhere for 2 h. Then, cells were washed with fresh medium and incubated with HPE in DMEM. After a 24-h incubation, cells were washed, and 50 μL FBS-free medium containing 5 mg/mL MTT were added. The medium was discarded after a 4-h incubation at 37 °C, and formazan blue formed in the cells was dissolved in DMSO. The absorbance at 540 nm of MTT-formazan of untreated cells, measured in a microplate reader (Bio-RAD, Hercules, CA), was taken as 100% of viability.

Nitrite determination

Nitrite accumulated in the culture medium was measured as an indicator of NO production, according to the Griess reaction. Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine–HCl], incubated at room temperature for 10 min, and then, the absorbance at 550 nm was measured in a microplate reader. Fresh culture medium was used as the blank. The amount of nitrite in the samples was evaluated by referring to a sodium nitrite serial dilution standard curve.

Scavenging of NO

Stock solutions of 100 mM sodium nitroprusside (SNP) were prepared in PBS that had been bubbled with argon, immediately before the assay. SNP solutions (50 μL) were added to 950 μL of HPE in either PBS or culture medium. Solutions were incubated at 25 °C for 2.5 h. Aliquots (50 μL) were analyzed for nitrite as described above.

Enzyme-linked immunosorbent assay (ELISA)

PGE_2 and TNF-α levels in the macrophage culture medium were quantified using EIA kits (eBIOSCIENCE, San Diego, CA) according to the manufacturer's instructions.

Western blot analysis

RAW 264.7 cells were rinsed twice with ice-cold PBS and harvested by scraping in 200 μL well ice-cold hypotonic lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 1.5 μg/mL soybean trypsin inhibitor, 7 μg/mL pepstatin A, 5 μg/mL leupeptin, 0.1 mM benzamidine, and 0.5 mM DTT), and incubated for 15 min on ice. The lysates were centrifuged at 13,000*g* for 5 min, and supernatants (cytosolic fraction) were immediately portioned and stored at −80 °C up to two weeks. The nuclear pellet was re-suspended in 60 µL of high-salt extraction buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF, 1.5 µg/mL soybean trypsin inhibitor, 7 µg/mL pepstatin A, 5 µg/mL leupeptin, 0.1 mM benzamidine, and 0.5 mM DTT) and incubated with shaking at 4 °C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000*g*, and supernatant was used for Western blot analysis using anti-iNOS, anti-COX-2, anti-p65, or anti-IκB-α monoclonal antibodies. The blots were washed two times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated anti-IgG antibody for 1 h at room temperature. Blots were again washed five times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA). All membranes were stripped and reprobed for β-actin or PARP as control. The immune-complex was quantified by densitometric scanner.

Measurement of intracellular reactive oxygen species (ROS) generation

DCFDA in PBS (10 µM) was added in the medium 30 min before ending the treatment of RAW 264.7 cells to label intracellular ROS. Then, the medium was removed, and the cells were washed with PBS, re-suspended in the same buffer, and immediately subjected to fluorescence-activated cell sorting (FACS) analysis (Coulter® Epics® XL™, Beckman).

Statistical analysis

One-way ANOVA was used to determine differences between different concentrations or treatments. When significant values were found ($p < 0.05$), post hoc comparisons of the means were made using Fisher's test. Differences were analyzed using Minitab Software (Minitab, State College, PA, USA).

Results

Following exposure to LPS, macrophages release a number of inflammatory mediators including NO, prostaglandin E_2, and cytokines. With respect to un-stimulated RAW 264.7 macrophages, the release of nitrite, a stable metabolite of NO, remarkably increased after a 24-h LPS treatment. The production of NO was suppressed in a dose-dependent manner when macrophages were pre-incubated with HPE (1- to 20-mg fresh nut equivalents/mL of cell medium) for 1 h, before adding LPS and subsequently co-incubating for 24 h (Fig. 1a). A 24-h exposure to the highest HPE concentration did not affect cell viability (not shown), ruling out that the decrease in the mediator was caused by cytotoxicity. Interestingly, quite comparable suppressive effects on the NO production were observed when the cells were exposed to HPE for 1 h, washed, and then incubated with LPS in fresh medium for 24 h (Fig. 1a), providing evidence that a brief pre-treatment of the cells was enough to make cells more resistant to the subsequent LPS injury. Then, this experimental procedure was adopted to carry out the entire study. HPE did not modify the amount of nitrite generated from the spontaneous decomposition of SNP in a cell-free system, showing that extract components did not possess any ability to scavenge NO, at least at the assayed amounts (Fig. 1b).

RAW 264.7 macrophages LPS-stimulated for 24 h released amounts of TNF-α and PGE_2 higher than un-stimulated cells. Pre-treatment of the cells with HPE dose dependently inhibited the release of both mediators, though release of PGE_2 was inversely related to the HPE amount, and the higher the concentration the lower the effect (Fig. 2).

Production of NO and PGE_2 in stimulated macrophages is to be ascribed to activity of the inducible iNOS and

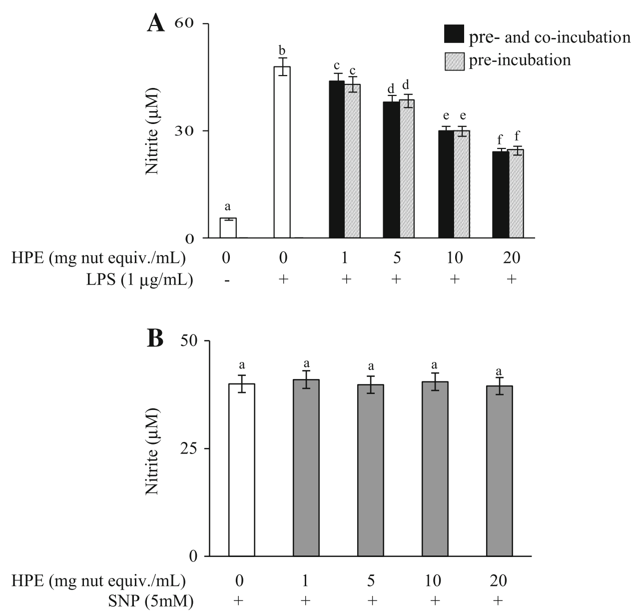

Fig. 1 NO production from LPS-treated RAW 264.7 cells (**a**) or from SNP in cell-free culture medium (**b**) and effect of HPE. (**a**) Cells were pre-incubated for 1 h with either vehicle or HPE in DMEM as showed in figure. Then, either LPS was added and incubation protracted for 24 h at 37 °C in air-5% CO_2 atmosphere (*black bars*), or the medium was replaced with fresh DMEM and cells stimulated with LPS (*dashed bars*). (**b**) SNP at 5 mM was incubated in DMEM for 2.5 h at 25 °C in the absence (*white bar*) or in the presence of HPE (*gray bars*). NO was measured as nitrite by Griess reaction as reported in methods. Each value is the mean ± SD of three or four experiments carried out in triplicate. In each panel, values not sharing the same letter were significantly different ($p < 0.05$, Fisher's test)

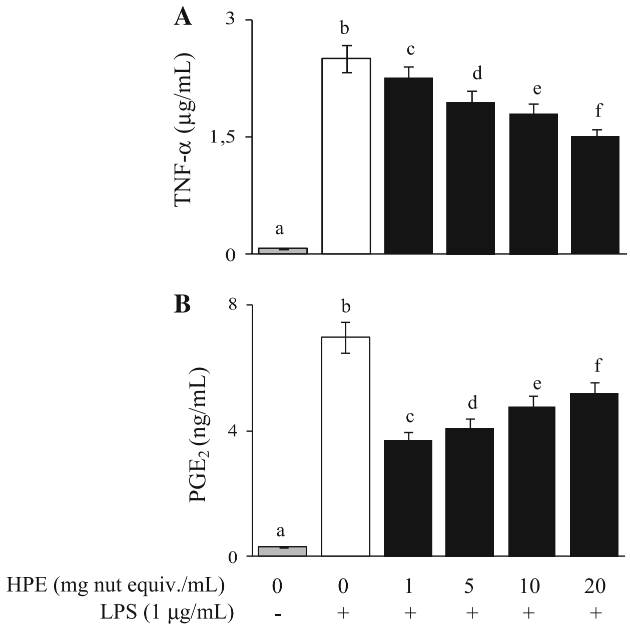

Fig. 2 Inhibition of LPS-induced TNF-α (**a**) and PGE_2 (**b**) production by HPE in RAW 264.7 cells. Cells were pre-incubated for 1 h with vehicle (*white bar*) or HPE (*black bars*) in DMEM. Then, the medium was replaced with fresh DMEM, and the cells stimulated with LPS for 24 h at 37 °C in air-5% CO_2 atmosphere. Each value is the mean ± SD of three or four experiments carried out in triplicate. In each panel, values not sharing the same letter were significantly different ($p < 0.05$, Fisher's test)

COX-2, respectively. RAW 264.7 cells did not express detectable amounts of either iNOS or COX-2 proteins when incubated in the absence of LPS for 24 h, whereas the level of both enzymes increased upon LPS activation (Fig. 3). Pre-treatment with HPE caused suppression of both enzymes, with the inhibitory effects on the COX-2 levels inversely related with HPE amounts (Fig. 3).

Exposure of macrophages to LPS is followed by phosphorylation and degradation of the inhibitor I$\kappa\beta$-α. This allows the activation of NF-κB with translocation of its p65/p50 portion from cytosol into the nucleus [27]. In comparison with LPS-activated cells, HPE pre-treatment led to a significant dose-dependent decrease in the nuclear p65 subunit (Fig. 4a), whereas higher amounts of the inactive cytosolic subunit were evident (Fig. 4b), indicating that NF-κB activation was prevented.

Oxidant-induced signaling is associated with the inflammatory response in macrophages. ROS production in RAW 264.7 cells after exposure to LPS for 60 min is shown in Fig. 5. Pre-treatment of cells with varied amounts of HPE for 1 h prior LPS activation, resulted in a net dose-dependent decrease in the mean fluorescence intensity (MFI) of the DCFDA-labeled cells, indicating that ROS production was inhibited.

The Sicilian pistachio nut is a good source of isoflavones and proanthocyanidins [24]. The amounts of genistein and daidzein in the HPE preparation in our hands were 1.27 and 2.06 mg/100 g fresh pistachio nut, respectively. Fractionation of proanthocyanidins according their polymerization degree resulted in the isolation of an oligomeric fraction (OF) and a polymeric one (PF), amounting to 25- and 220-mg cyanidin equivalents/100 g fresh pistachio nut, respectively. To investigate active HPE components, the anti-inflammatory effect of either isoflavones or individual proanthocyanidin fractions was assessed taking into account their amount in HPE (20-mg fresh nut equivalent/mL). Whereas a 1-h pre-treatment of RAW 264.7 macrophages with 1 μM genistein and/or 1.6 μM daidzein did not affect the LPS-stimulated formation of either NO or TNF-α or PGE_2, pre-treatment of the cells with either OF (0.02 μM) or PF (0.15 μM) inhibited the release of the pro-inflammatory mediators, with PF showing the highest effect (82 to 90% of the HPE effect) (Fig. 6). A combination of both genistein and daidzein (1 and 1.6 μM, respectively) and HPE (20-mg fresh nut equivalents/mL) did not cause an inhibitory effect higher than that of HPE alone (not shown). In other assays, the formation of PGE_2 was observed in cells pre-treated with amounts of OF and PF relevant to 1-mg fresh nut equivalents/mL HPE and compared with HPE. While OF did not show a significant effect, PF inhibited PGE_2 formation to the extent of 85% than HPE (Fig. 6, inset). Finally, the level of phosphorylated I$\kappa\beta$-α in LPS-stimulated 264.7 RAW macrophages, after pre-treatment with either HPE or the isolated PF was evaluated. Both pre-treatments resulted in a remarkable reduction in the LPS-induced phosphorylation of I$\kappa\beta$-α, with an inhibitory activity of PF that was 70% of HPE (Fig. 7).

Discussion

Pistacia vera hydrophilic extract affects inflammatory pathways in RAW 264.7 macrophages

Overproduction of NO and overexpression of TNF-α in activated monocytes are key events in initiating and amplifying an inflammatory process [28], and their level may provide a first reliable indication of the eventual effect of treatments. In our model, 1-h pre-treatment of RAW 264.7 macrophages with non-toxic amounts of HPE, followed by washing of cells before stimulation with LPS, caused a dose-dependent decrease in NO and TNF-α released. In addition, the LPS-induced production of the prostanoid mediator PGE_2 [29, 30] was also inhibited, though the higher the HPE amount the lower the effect. Pre-treatment of RAW macrophages with HPE resulted in a specific inhibition of the LPS-induced expression of iNOS and COX-2, the enzyme isoforms responsible for the

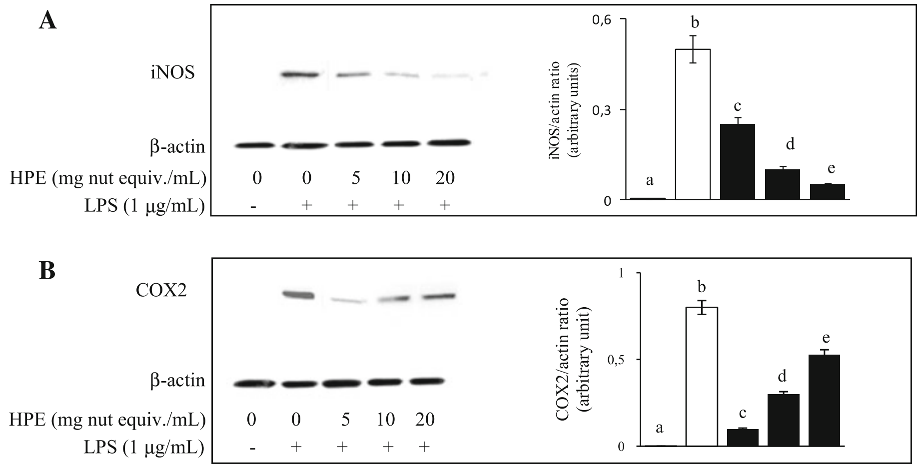

Fig. 3 Effect of HPE on LPS-induced iNOS (**a**) and COX2 (**b**) expression in RAW 264.7 cells. Cells were pre-incubated for 1 h with vehicle or HPE in DMEM. Then, the medium was replaced with fresh DMEM, and the cells stimulated with LPS for 24 h at 37 °C in air-5% CO_2 atmosphere. Cell lysates were submitted to electrophoresis, and the expression levels of the proteins were detected with specific antibodies. *β*-actin was used as an internal control. Each *panel* shows representative Western blot analyses with densitometric analyses. The value is the means ± SD of four separate experiments. Values not sharing the same letter were significantly different ($p < 0.05$, Fisher's test)

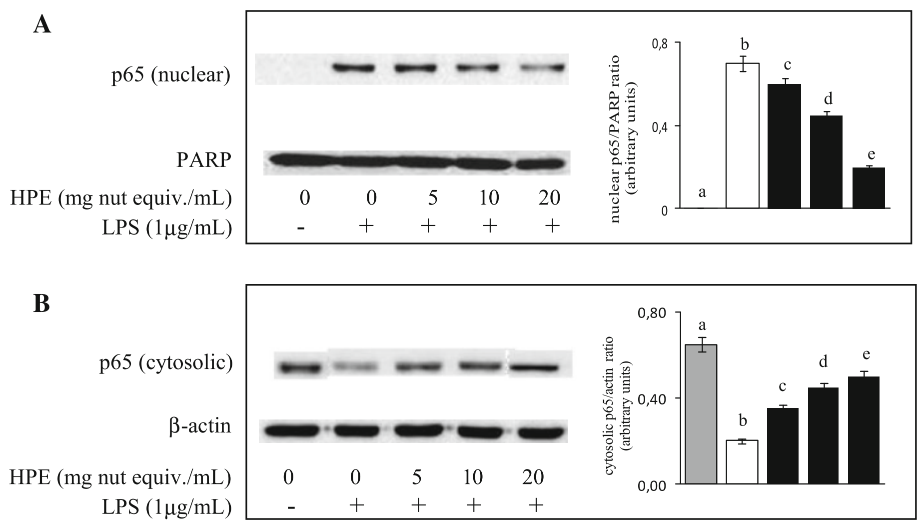

Fig. 4 Effect of HPE on LPS-induced p65 translocation in RAW 264.7 cells. Cells were pre-incubated for 1 h with vehicle or HPE in DMEM. Then, the medium was replaced with fresh DMEM, and the cells stimulated with LPS for 30 min at 37 °C in air-5% CO_2 atmosphere. Nuclear (**a**) and cytosolic (**b**) fraction was prepared, submitted to electrophoresis, and the levels of the proteins detected with specific antibodies. PARP and *β*-actin were used as internal control for nuclear and cytosolic fraction, respectively. Each *panel* shows representative Western blot analyses with densitometric analyses. The values are the means ± SD of four separate experiments. Values not sharing the same letter were significantly different ($p < 0.05$, Fisher's test)

production of NO and PGE_2, respectively. Whereas the iNOS expression was suppressed dose dependently, the suppressive effects on COX-2 appeared inversely related to the HPE amount. Different pathways control the expression of iNOS or COX-2. Whereas the main regulatory step for the iNOS is the activation of the transcription factor NF-κB [28], additional factors regulate the gene expression for COX-2 [31]. Moreover, COX-2 is also affected post-transcriptionally at the level of mRNA stability, and its activity is known to be affected by NO [31]. Then, in spite of the global anti-inflammatory effect, it may be not surprising that active components of HPE may influence to a different extent different potential regulatory targets. It should be noted that the inhibition of the nitrite release did not appear as a result of a direct scavenging by HPE components, suggesting that modulation of the iNOS expression was a main regulatory step to account for the decrease in NO in our system.

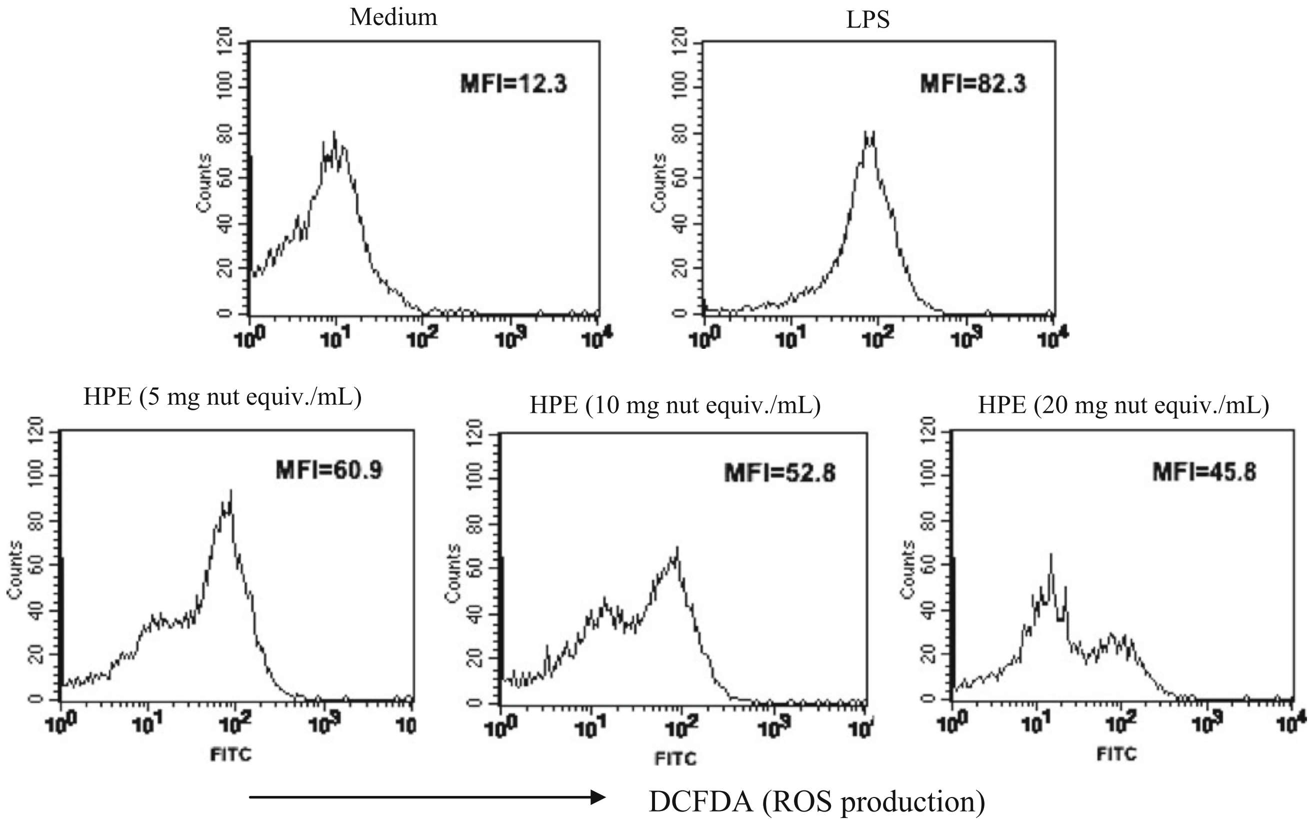

Fig. 5 Inhibition of LPS-induced ROS production by HPE in RAW 264.7 cells. Cells were pre-incubated for 1 h with vehicle or HPE in DMEM. Then, the medium was replaced with fresh DMEM, and the cells stimulated with LPS for 60 min at 37 °C in air-5% CO_2 atmosphere; 30 min before the end of incubation, 10 μM DCFDA was added. Mean fluorescence intensity (MFI) was measured using flow cytometry. The experiment was repeated four times with similar results

Various interconnected signal transduction pathways concur to macrophage activation, with the pro-inflammatory NF-κB playing a central role in orchestrating the response to a wide range of insults, including LPS [27, 32]. In resting cells, the most abundant form of NF-κB is the cytosolic inactive p65/p50 heterotrimer, with the p65 subunit, containing the transcriptional activation domain, bound to the IκB inhibitory protein. Activation of macrophages results in phosphorylation and degradation of IκB followed by nuclear translocation of the p65/p50 dimer and its binding to specific response elements in the DNA [27]. In our study, the remarkable increase in the nuclear p65 subunit consequent to LPS stimulation was inhibited dose dependently by HPE pre-treatment of RAW 264.7 macrophages. Considering that NF-κB, in synergy with other transcriptional activators, coordinates the gene expression for pro-inflammatory enzymes and cytokines, including iNOS, COX-2, and TNF-α [33], these findings suggest that anti-inflammatory activity of HPE is at least in part mediated by inhibition of the NF-κB activation pathway.

Oxidant stress plays a major role in several aspects of acute and chronic inflammation. Signaling pathways leading to cytosolic NF-κB activation, as well as nuclear activity of NF-κB, are under control of the cell redox state and are finely modulated by oxidants [34]. External stimuli such as LPS, or physiological activators, may concur to increase ROS in activated macrophages [35]. According to recent research TLR-4, either activated through pathogens or in response to damage-associated molecular patterns start an inflammatory response with early production of ROS [35] through direct interaction with the membrane NADPH oxidase [34, 36–38]. We found that pre-treatment of RAW 264.7 macrophages with HPE before LPS activation resulted in a net dose-dependent decrease in intracellular ROS generated. Taken together, our findings suggest that by affecting the redox state of LPS-activated RAW 264.7 cells, HPE component(s) may affect redox-sensitive signal transduction pathways thereby modulating the NF-κB activity and finally downregulating the expression of iNOS, COX-2, and TNF-α. Other molecular pathways involving LPS-sensitive factors such as C/EBP and fos/jun cannot be ruled out. Further studies will clarify whether additional mechanisms, besides the NF-κB-centered signaling cascade, may be involved in the anti-inflammatory activity of pistachio nut extract.

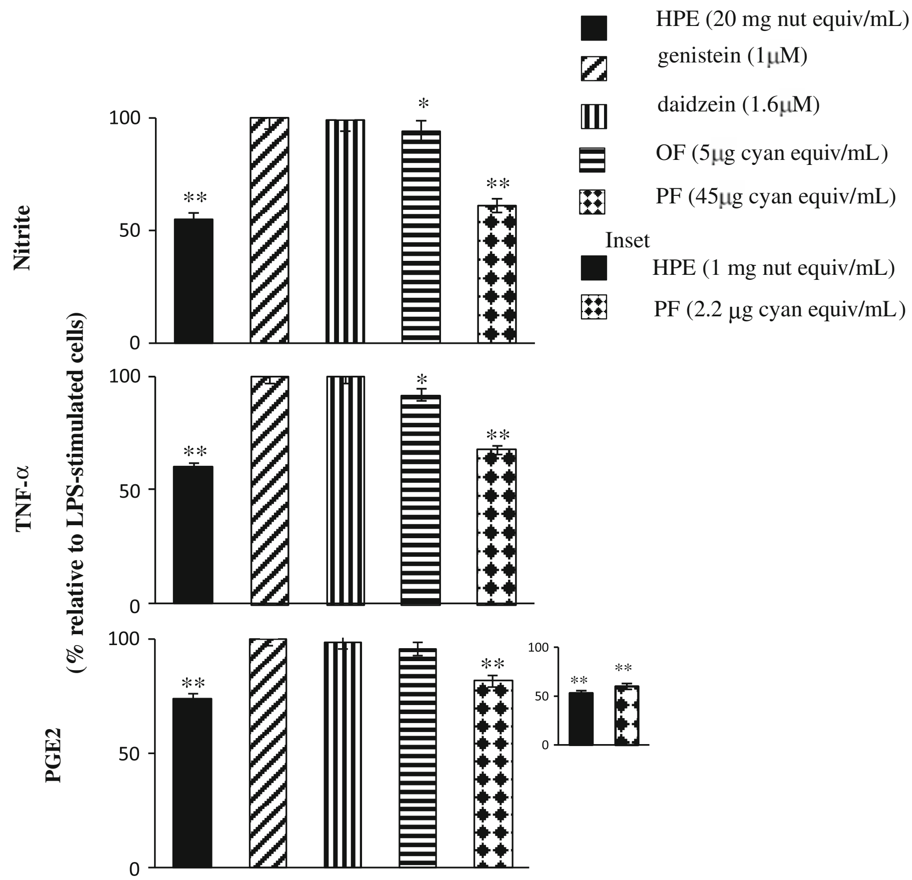

Fig. 6 Effect of isoflavones, proanthocyanidin oligomer fraction (OF), or proanthocyanidin polymeric fraction (PF) from HPE (20 mg nut equiv/mL) on the LPS-induced release of NO, TNF-α, and PGE_2 as compared with HPE. *Inset* shows the effect of PF from HPE (1 mg nut equiv/mL) on release of PGE_2 as compared with HPE. Cells were pre-incubated for 1 h with HPE or with individual compounds or fraction, in DMEM. Then, the medium was replaced with fresh DMEM, and the cells stimulated with LPS for 24 h at 37 °C in air-5% CO_2 atmosphere. Values are expressed as percentage of the value measured in LPS-activated Raw 264.7 macrophages and are the mean ± SD of three–four experiments. With respect to value measured in LPS-activated Raw 264.7 macrophages, values are significant with p: * < 0.05; ** < 0.01 (Student's t test)

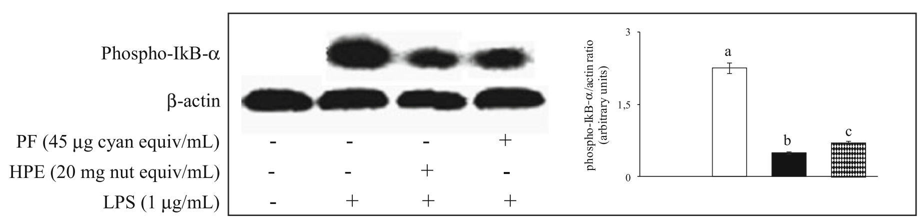

Fig. 7 Effects of PF on the phosphorylation of IκB-α in LPS-treated RAW 264.7 cells as compared with HPE. Cells were pre-incubated for 1 h with vehicle, HPE, or PF in DMEM. Then, the medium was replaced with fresh DMEM, and the cells stimulated with LPS in the presence of the proteosome inhibitor MG-132, added at 50 μM, for 30 min at 37 °C in air-5% CO_2 atmosphere. Cell lysates were submitted to electrophoresis, and the protein was detected with specific antibody. β-actin was used as an internal control. *Panel* shows representative Western blot and densitometric analysis. The value is the means ± SD of three separate experiments. Values not sharing the same letter were significantly different ($p < 0.05$, Fisher's test)

Anti-inflammatory activity of HPE components

Proanthocyanidins and isoflavones are the major bioactive phytochemicals in our HPE preparation [24]. Interferences of isoflavones with basic mechanisms of inflammation in macrophages have been reported [39, 40]; however, under our experimental setup, these molecules, either alone or combined, did not appear to concur to the anti-inflammatory effects of HPE. Moreover, a combination of both isoflavones and HPE did not result in an inhibitory effect higher than that of HPE alone, which also ruled out interactions of the isoflavones with other extract components. Concentration is possibly a crucial issue. Indeed, genistein at 20 μM has been reported to inhibit NO and PGE_2 production of LPS plus INF-γ in activated macrophages [39], and it has also been shown to have strong anti-inflammatory activity in LPS-stimulated monocyte-derived dendritic cells at 200 μM [40]. On the other hand, consistent with our findings, no activity of daidzein in the low micromolar range was observed in the study mentioned before [39].

Anti-inflammatory effects of proanthocyanidins have been reported in rats and mice [41], in human studies [42], as well as in inflammatory cell models [43–45]. In our study, HPE proanthocyanidins either oligomers or polymers were found to inhibit the LPS-induced release of NO,

PGE_2, and TNF-α, with oligomers poorly effective with respect to polymers that contributed almost entirely to the HPE activity. These data may be the reflection of the amounts of proanthocyanidins assayed and/or indicate a different ability of oligomers and polymers to affect the macrophage response, as observed in other researches [44, 46–49]. To further highlight the role of these components, PF was also found almost effective as the whole HPE in inhibiting phosphorylation, and then activation, of IκB-α.

How the HPE proanthocyanidins may modulate the LPS-stimulated NF-κB activation in our cell model may be object of speculation. In spite of high hydrogen-donating abilities and propensity for nitration, beneficial effects of proanthocyanidins may result from modes of action other than acting as antioxidants [50]. The amphiphilicity of these compounds and the ability to partition into [51] and interact with both membrane lipids and proteins are considered a major determinant of their pharmacological activity [52]. In addition, binding and neutralization of LPS by proanthocyanidins have also been shown [53]. In our cell system, direct interference with LPS seems to be ruled out, since pre-treatment of macrophages with the active compounds was followed by washing of cells before stimulation. Modulation and interplay of surface receptors and adaptor proteins are key events of the macrophage response to LPS [34, 54, 55]. The recently observed modulation of TLR-4 activation by flavonoids [56], and the interaction of proanthocyanidins with NADPH oxidase [57], may shed some light on the activity of these compounds as signaling molecules. Partition coefficients measured for proanthocyanidins [51] indicate that molecules at higher polymerization degree are more likely to partition into the lipid phase, which could facilitate interaction with membrane components [52]. Interestingly, in contrast to our findings, proanthocyanidins from grape seeds that exhibited an anti-inflammatory activity when co-incubated with activated RAW 264.7 macrophages failed to decrease formation of PGE_2, when were only pre-incubated [44], suggesting peculiar activities of pistachio proanthocyanidins.

Conclusive remarks

Data presented demonstrate that a hydrophilic extract from *P. vera* effectively inhibits the LPS-induced inflammatory response in RAW 264.7 macrophages, through modulation of the NF-κB activation pathway and that highly polymeric proanthocyanidin components substantially parallel the activity of the whole extract. Since only flavonol monomers and dimeric proanthocyanidins are absorbed [58–61], systemic effects of proanthocyanidins would be limited to these compounds or eventually to not yet characterized metabolites and/or colonic degradation products from the polymeric molecules [58]. In this context, our findings cannot help to rationalize the cardiovascular protection nor provide a molecular interpretation for the anti-inflammatory effects of pistachio nut diets [20]. Eventual beneficial effects of polymeric proanthocyanidins could be considered at the gastrointestinal level [58, 62]. A permanent actively controlled and downregulated physiological inflammation characterizes the intestinal mucosa due to the intense exchanges between the intestinal microflora and the mucosal immune system. In addition, the latter has to avoid inflammatory reactions toward harmless antigenic structures of alimentary origin. A single serving of pistachio nut (28.34 g) [63] contains around 70-mg proanthocyanidins. Once diluted in a gastrointestinal volume of 600 mL [64], this results in a 0.40 mM concentration (as cyanidin equivalents), of the same order as the concentrations selected in our cell model. At any instance, further studies are required to assess whether and how much pistachio nut proanthocyanidins are affected by the digestion process to establish real amounts and molecular composition of pistachio digesta at the level of intestinal epithelium. These investigations are currently performed in our laboratory.

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