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In vitro anti-inflammatory activity of phenolic rich extracts from white and red common beans

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ABSTRACT

According to epidemiological evidence, diets rich in fruits and vegetables can reduce the incidence of several chronic diseases that share an inflammatory component. These protective effects are attributed, in part, to the occurrence of different antioxidant components, mainly phenolic compounds. Our aim was to characterise phenolic composition, and to determine antioxidant and anti-inflammatory activities of phenolic rich extracts obtained from two kinds of common beans, white kidney beans (WKB) and round purple beans (RPB). Phenolic acids were the predominant component in WKB extracts, whereas RPB extracts presented higher concentrations of phenolic compounds, mainly catechin derivatives, proanthocyanidins and catechin glucoside. In addition, RPB extracts showed higher antioxidant capacity and higher anti-inflammatory activity by the reduction of NO production and cytokine mRNA expression of LPS stimulated macrophages. These results suggest that common bean extracts may be used as a source of anti-inflammatory agents as well as a dietary complement for health promotion.

1. Introduction

Legumes can be considered as an important component of healthy diets not only due to their nutritional value (high content in proteins and low content in fat), but also because of their functional properties. Consumption of pulses has previously been associated with a reduced risk of cardiovascular diseases, diabetes and even certain types of cancer (Curran, 2012; Hutchins, Winham, & Thompson, 2012). Apart from soy beans, white and red beans are some of the most consumed legumes, being part of the typical diet in several western countries. The functional components of these legumes include complex carbohydrates, soluble fibres, essential vitamins, phytate, lectins, and polyphenols. Among all of them, phenolic compounds including flavonoids and isoflavones have emerged as potent antioxidant molecules (Rahman, Biswas, & Kirkham, 2006). Epidemiological studies have demonstrated positive correlations between the consumption of foods with a high content in phenolic substances and high antioxidant values, as well as a decrease in the incidence of several diseases, such as cancer, ageing and cardiovascular diseases (Liu, 2013). Moreover, phenolic compounds have attracted great attention during the last few years due to the large variety of biological activities that they exhibit, such as anti-inflammatory, anti-tumour or anti-atherogenic activities. Many of these effects cannot be explained solely on the basis of their antioxidant properties. Investigations into the mechanisms of action of these molecules have demonstrated that polyphenols, besides acting as free radical scavengers, can also modulate cellular signalling processes during inflammation (García-Lafuente, Guillamón, Villares, Rostagno, & Martínez, 2009).

Inflammation is a physiological process that initiates in response to bacterial infection or tissue damage. Macrophages are the first line of defense of the immune system against noxious agents. Inflammatory stimuli such as lipopolysaccharide (LPS) activate macrophages to produce a variety of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukins (IL-1β, or IL-6), as well as other inflammatory mediators including prostaglandin E_2 (PGE_2) and nitric oxide (NO), which are synthesised by cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS), respectively. Production of these mediators has been demonstrated in several inflamed tissues, along with enhanced expression of their mRNAs, and they are involved in the pathogenesis of several diseases, such as atherosclerosis, obesity, metabolic syndrome, diabetes, neurodegenerative diseases, and several types of cancers (Cardona, Garcia, & Cardona, 2013; Lee, Han, Nam, Oh, & Hong, 2010; Pradhan, 2007). Molecules that reduce the expression

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of these proinflammatory genes could be useful in the treatment of many chronic diseases with an underlying inflammatory origin.

In the present work, a mouse macrophage cell line RAW 264.7 stimulated with LPS was used, as an *in vitro* model, to investigate the anti-inflammatory activity of phenolic rich extracts obtained from two kinds of legumes commonly found in the human diet: red beans and white beans.

2. Materials and methods

2.1. Samples

Samples of beans were purchased from IGP Judía de El Barco de Ávila (Spain). They were a red bean called round purple bean (RPB), and a white bean called white kidney bean (WKB), both of them being *Phaseolus vulgaris*. They were stored at −20 °C until used to obtain methanolic extracts.

2.2. Chemicals and reagents

HPLC (high-performance liquid chromatography) grade methanol, acetonitrile and sodium carbonate were obtained from VWR-International (Darmstadt, Germany) while acetic acid (96%) and Folin–Ciocalteau reagent were purchased from Merck (Darmstadt, Germany). The 2,2′-diphenyl-1-picryhydrazyl (DPPH) was from Cayman Chemical Company (Ann Arbor, Michigan USA). Ultra pure water was supplied by a Milli-Q water Advantage A10 purifier system from Millipore (Bedford, MA, USA). The standards, sinapic, trans-*p*-coumaric and trans-ferulic acids, flavanones naringenin and hesperitin, flavonol quercetin, flavanol (+)-catechin and anthocyanidin malvidin 3-O-glucoside chloride, were obtained from Extrasynthèse (France), and gallic acid from Merck (Darmstadt, Germany). Fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), Dulbecco's modified Eagle's minimum essential medium (DMEM), foetal bovine serum (FBS), penicillin, streptomycin, Griess reagent, dimethyl sulfoxide (DMSO), MTT reagent (thiazolyl blue tetrazolium bromide), lipopolysaccharide (LPS) (*Escherichia coli*, serotype 0111:B4), and other chemicals were obtained from Sigma–Aldrich Co. (Saint Louis, MO, USA). Reagents for DNA extraction and purification and PCR were purchased from Applied Biosystems (Foster City, CA, USA) and antibody anti-NF-κB-p65 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.3. Extraction of polyphenolic compounds from beans

The protocol used to obtain polyphenolic rich extracts from bean samples was based on the method previously optimised by Rostagno, Palma, and Barroso (2003). Briefly, the ultrasound-assisted extraction method consisted of 3 consecutive extractions of 30 g of sample; firstly the sample was extracted with 250 ml of 50% methanol, then with 250 ml of 80% methanol, and finally with 50 ml of 100% methanol. The 3 extractions were carried out for 30 min at 60 °C. After each extraction step, the sample was centrifuged at 10 °C for 10 min at 4000 rpm on a Universal 320R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), the supernatant collected and the solid submitted to the following extraction step. Extractions were carried out on a multi-frequency (25 and 45 kHz) ultrasonic bath (Transsonic TH-I-55, Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) operating at 25 and 45 kHz (alternatively) at 100% intensity output. After the last extraction, supernatants were combined. The liquid extract was evaporated to half volume under vacuum, using a rotary evaporator (Laborota 4000 efficient, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 45 °C. After evaporation, the same volume of water as the evaporated liquid was added and then freeze-dried. The whole extraction was repeated until the necessary amount of polyphenolic rich extract for the assays was obtained. Freeze-dried extracts were reconstituted in 50% aqueous methanol for the HPLC analysis and the Folin–Ciocalteau, DPPH and ORAC assays, and reconstituted in culture medium (DMEM) and filtered through a 0.2 μm nylon syringe filter (VWR-International, Darmstadt, Germany), before the cellular treatments.

2.4. High performance liquid chromatography

2.4.1. Liquid chromatography diode array detection (HPLC–PAD)

High-performance liquid chromatography (HPLC) was conducted by using a Waters HPLC system (Waters, Milford, Mass, USA) equipped with a 717 plus autosampler, a quaternary pump 600-MS controller and a 996 photodiode-array detector (PAD). Separation of phenolic compounds was based on a previous method from Dueñas, Hernández, Estrella, and Rabanal (2003). A reverse phase C18 column (Nova-Pak, 300 × 3.9 mm, particle size 4 μm) was used. The mobile phase was comprised of two solvents, A: water/acetic acid (98:2, v/v) and B: water/acetonitrile/acetic acid (78:20:2, v/v/v). The gradient profile was 0 min, 100% A; 55 min, 20% A; 57 min, 10% A; 70 min, 10% A; 80 min, 5% A; 90 min, 0% A; 120 min, 0% A. Flow rate was 0.70 ml/min. The column was cleaned after gradient and between injections with 4 min of 100% acetonitrile with a flow rate of 0.7 ml/min, and re-equilibrated for 15 min with the initial conditions. Injection volume was 50 μl. Detection was performed by scanning from 210 to 400 nm with an acquisition speed of 1 s. The samples were analysed in duplicate. The software for the equipment control and data acquisition was Empower 2 version 6.10.01.00.

2.4.2. Liquid chromatography/electrospray mass spectrometry (HPLC–ESI-MS)

Mass spectra were obtained using a Hewlett Packard 1100MS (Palo Alto, CA) chromatograph equipped with an API source, using an ESI interface. The solvent gradient and column used were the same as for HPLC–PAD. ESI conditions were as follows: negative mode, nitrogen was used as the 40 psi nebulizing pressure drying gas, 10 l/min at 340 °C; voltage at capillary entrance, 4000 V; and variable fragmentation voltage, 100 V ($m/z < 200$), 200 V (m/z 200–1000), 250 V (m/z 1000–2500). Mass spectra were recorded from m/z 100 to m/z 2500.

2.5. Identification and quantification of phenolic compounds

Chromatographic peaks were identified by comparison of their retention times, UV spectra and data of UV spectral parameters with those of the standards and confirmed by HPLC–MS. Other compounds, for which standards were not available, and which presented an UV spectrum similar to hydroxycinnamic acids, procyanidins, anthocyanins and flavonols, were identified as derivatives of them and were confirmed by HPLC–MS (ESI) or by the data of UV spectral parameters obtained in studies previously developed (Dueñas et al., 2003).

Quantification was carried out using the external standard method with commercial standards. The calibration curves were made by injection of different volumes of the stock solutions along the range of concentration observed for each compound, using a linear regression for the relationship of sum area versus concentration obtained under the same conditions used before for the samples analysed. The unknown non-flavonoid and flavonoid derivatives were quantified by using the calibration curves of the most similar compounds, and each concentration is expressed as amount of this similar compound. Regression equations and

correlation coefficient (R^2) were calculated using Microsoft Excel 2007 software.

2.6. Polyphenol content determined by Folin–Ciocalteau assay

Total phenolic content (TPC) of extracts were determined by Folin–Ciocalteu assay using gallic acid as the standard (Singleton & Rossi, 1965). Sample (750 μl), distilled water (4.75 ml), Folin–Ciocalteu reagent solution (500 μl), 20% sodium carbonate (2 ml) and distilled water (2 ml) were vortexed and incubated for 30 min at room temperature.

The absorbance of the samples was measured at 750 nm using a microplate UV–Visible spectrophotometer (Fluostar Omega BMG, Labtech Int'l Ltd, East Sussex, UK) against distilled water as a blank. The total phenolic content was expressed as gallic acid equivalents (mg of GAE/g sample). The linear range of the calibration curve was 0–100 μg/ml (R^2 = 0.99).

2.7. Antioxidant activity determined by DPPH assay

The antioxidant activity, expressed as half maximal effective concentration (EC_{50}) of the extracts, was determined by reaction with the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical (Brand-Williams, Cuvelier, & Berset, 1995). The reaction was carried out with 2 ml of a methanol solution of DPPH (0.025 g/l) and solutions of different concentrations adequate for each sample. The absorbance was measured at 1 min intervals at 515 nm, until the reaction reached a plateau (time at the steady state). The percentage of remaining DPPH (% DPPHrem) was calculated as follows: % DPPHrem = [(Abs: 515 nm)sample/(Abs: 515 nm)control] × 100. This percentage was plotted against the sample concentration to obtain the EC_{50}, defined as the amount of antioxidant (mg of sample) necessary to decrease absorbance by 50%. The lower the EC_{50} value, the higher the antioxidant activity obtained. We used $1/EC_{50}$; a higher $1/EC_{50}$ indicates higher antioxidant activity.

2.8. Antioxidant activity established by ORAC assay

Antioxidant activity was measured by oxygen radical absorbance capacity (ORAC) assay (Dávalos, Gómez-Cordovés, & Bartolomé, 2004). Samples (20 μl) were dispensed in a 96-well microplate (NUNC A/S, Roskilde, Denmark) along with standard Trolox solutions and blanks, and loaded into a microplate reader, which was programmed to inject each well with 120 μl fluorescein and 60 μl AAPH, and to record the fluorescence of the mixture every minute for 104 min (λex = 485 nm, λem = 520 nm). Antioxidant activity of the extracts was expressed as μmol Trolox equivalents/g of sample, and was calculated from the regression equation of Trolox concentrations and net area under the fluorescence decay curve, which was generated by FLUOstar Optima software (V2.10 R4, BMG Labtech Inc., Durham, NC).

2.9. Cell line

The macrophages cell line RAW 264.7 was obtained from the European Collection of Cell Cultures (ECACC) and cultivated in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a 5% CO_2 humidified atmosphere (CO_2 incubator, Heal Force). Cells were treated with the beans extracts at different concentrations during 1 h and stimulated with LPS (1 μg/ml) for the indicated period.

2.10. MTT – assay for measuring cell viability

Cells were seeded at a density of 10^4 cells/well in 96-well plates overnight, and pre-treated with different concentrations of the extracts (0.625, 1.25, and 2.5 mg of extract/ml) for one hour, before stimulation with LPS. RAW 264.7 viability was measured after 24 h of exposure to the tested extracts with a colorimetric assay based on the ability of mitochondria in viable cells to reduce MTT. The MTT solution was added at a concentration of 0.5 mg/ml into each well and, after 3 h of incubation at 37 °C, the medium was discarded and the formazan blue formed in the cells was dissolved in DMSO. Optical density at 570 nm was determined with a microplate reader. The optical density of formazan formed in LPS treated cells was taken as 100% of viability.

2.11. Nitrite determination

Cells were seeded onto 96-well plates with 2×10^5 cells/well and allowed to adhere overnight. Then, medium was removed and replaced with 0.2 ml of fresh medium either without foetal bovine serum, alone or containing different concentrations of extracts (0.625, 1.25, and 2.5 mg of extract/ml). After 1 h of incubation, LPS stimulation was performed. LPS was added at different concentrations (10 ng/ml, 100 ng/ml or 1 μg/ml) for 24 h. The cell-free culture medium was collected and 50 μl were used for NO determination. The nitrite accumulated in culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 50 μl of cell culture medium was mixed with an equal volume of Griess reagent (equal volumes of 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamide-HCl), incubated at room temperature for 10 min, and then the absorbance was measured at 550 nm using a microplate reader (FLUOstar Optima, BMG Labtech Inc., Durham, NC). The amount of nitrite present in the samples was calculated by means of a standard curve generated using serial dilutions of $NaNO_2$ in fresh culture medium.

2.12. Real time PCR of mRNA inflammatory mediators

2.12.1. RNA extraction and purification

Cells were plated onto 6 well plates at a density of 10^6 cells/well and incubated with extracts for 1 h prior to LPS stimulation. After 6 h, cells were lysed and total RNA extraction was performed by using the Ribopure kit from Applied Biosystems. The purified RNA was treated with DNAse (TURBO DNA-free kit from Applied Biosystems) and quantified by spectrophotometry at 260 nm. The purity of extracted RNA was measured by 260/280 index and it was in all the samples between 1.8 and 2.1. The RNA quality was assessed in each sample by capillary electrophoresis by using the Experion™ RNA StdSens Analysis Kit (Bio-Rad). The samples were shown to reach a RQI higher than 7.0, to ensure standards met the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments).

2.12.2. Reverse transcription

cDNA was synthesised from 0.5 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (AppliedBiosystems) in an Advanced Primus 96 thermocycler (PEQLAB).

Quantitative PCR was performed on the Step One System (Applied Biosystems, Foster City,CA, USA) using TaqMan Gene Expression Master Mix and pre-developed TaqMan Assays specific to mouse: iNOS (cat. No. Mm01309902_m1), TNF-α (cat. No. Mm99999068_m1), IL-1β (cat. No. Mm00434228_m1), IL-6 (cat. No. Mm99999064_m1), COX-2 (cat. No. Mm 00478374_m1), and for the housekeeping gene ACTB (cat. No. Mm01205647_g1). PCR was performed with TaqMan MGB probes labelled with FAM

Table 1
Total phenolic content and antioxidant activity of analysed extracts. All data have been obtained in triplicate.

Sample	Total phenolic compounds Folin–Cicalteau assay		Antioxidant activity DPPH assay		Antioxidant activity ORAC assay	
	mg Gallic acid equivalents/g sample	% RSD	$1/EC_{50}$	% RSD	μmol Equivalent Trolox/g sample	% RSD
WKB	4.63	2.71	109.14	0.03	217.97	15.12
RPB	13.41	0.47	171.06	0.20	304.24	9.39

reporter dye in a final reaction volume of 20 μl. The amplification conditions were the universal conditions described by the manufacturer. The probes were validated to ensure the amplification efficiency and linearity in preliminary experiments using different dilutions of the template. Expression values were obtained from Ct numbers detected by the Applied Biosystems analysis software. The target gene levels were expressed as the N-fold difference in the target gene expression relative to ACTB expression (ΔCt), where ΔCt was determined in each sample by subtracting the average Ct value of the target gene from the average Ct value of the ACTB; in addition, the relative gene expression was calculated as $2^{-\Delta Ct}$.

2.13. Western blot detection of NF-κB p65

RAW 264.7 cells were seeded in six-well plates at a density of 3×10^6 cells per well and cultured overnight. Cells were then pretreated with bean extracts (0.625 mg/ml) for one hour and stimulated with LPS (100 ng/ml) for 30 min. At the end of the treatment the cells were rinsed in phosphate buffered saline (PBS) twice and harvested by scraping. Cytoplasmic and nuclear proteins were extracted by using the NE-PER Nuclear and cytoplasmic extraction kit (Thermo Scientific). Both cytoplasmic and nuclear proteins were maintained at −80 °C for Western blot analysis. The protein concentration was determined using a Bradford protein assay reagent (Bio-Rad) and BSA as standard. Proteins were denatured in boiling water for 5 min and 10 μg of denatured proteins were loaded and separated by SDS–PAGE on a 10% polyacrylamide gel, electrically transferred to a PVDF membrane (Amersham Hybond-P) and probed with specific primary antibody anti-NF-κB-p65 (1:1000) from Santa Cruz Biotechnology (sc-372). The reactive bands were visualised with HRP-conjugated secondary antibody (1:5000) via ECL Prime Detection reagents (Amersham Biosciences).

3. Results

3.1. Phenolic composition and antioxidant activity of bean extracts

Table 1 shows total phenolic compounds, $1/EC_{50}$ values from DPPH assay and antioxidant activity from ORAC assay for white kidney beans (WKB) and round purple beans (RPB) extracts. RPB showed higher total phenolic compounds content than WKB and antioxidant activity values were greater for coloured beans than for white beans, independently of the assay used. The content in total phenolic compounds is consistent with the individual concentrations of phenolic compounds (Table 2) detected in both kind of beans; total concentration obtained by Folin–Ciocalteu assay and by HPLC–PAD–MS analysis are greater for RPB than for WKB.

The composition profile found for both bean extracts were quite different. In the case of WKB, the predominant phenolic

Table 2
Individual concentration of phenolic compounds in bean extracts analysed by HPLC–PAD–MS analysis. The samples were analysed in duplicate. Unknown non-flavonoid and flavonoid derivatives were expressed as amount of a similar compound.

Λ (nm)	MS fragments	Compound	Compound that is expressed	μg/g Freeze-dried extract	% RSD	μg/g Sample
WKB						
238, 294sh, 326	193, 385, 190.9	Feruroyl aldaric	Trans ferulic acid	270.95	15.04	34.68
312	190.9, 355	Coumaroyl aldaric	Trans coumaric acid	82.57	21.10	10.57
314	355, 190.9, 209	Coumaroyl aldaric	Trans coumaric acid	81.54	2.86	10.44
288, 326	385, 190.9	Feruroyl aldaric	Trans ferulic acid	440.41	7.99	56.37
327	385, 190.9, 192.9	Feruroyl aldaric	Trans ferulic acid	980.22	6.63	125.47
314	355, 190.9, 162.8	Coumaroyl aldaric	Trans coumaric acid	132.56	13.89	16.97
326	415, 385, 209.1, 190.9	Sinapic aldaric	Sinapic acid	352.88	11.07	45.17
330	415	Sinapic derivative	Sinapic acid	45.71	0.52	5.85
330	415	Sinapic derivative	Sinapic acid	1222.23	9.52	156.44
328	385, 190	Feruroyl aldaric	Trans ferulic acid	150.90	2.05	19.31
324	385, 209, 190	Feruroyl aldaric	Trans ferulic acid	169.93	12.74	21.75
330	415, 209	Sinapic acid	Sinapic acid	44.91	3.00	5.75
324	193	Ferulic acid	Trans ferulic acid	137.41	6.44	17.59
284	271, 609	Naringenin derivative	Naringenin	88.31	12.32	11.30
280, 330sh	301, 659	Hesperitin derivative	Hesperitin	1.12	11.77	0.14
RPB						
236, 278, 316	289, 451	Catechin glucoside	Catechin	2637.14	14.35	485.37
278, 319	355, 190.9	Coumaroyl aldaric	Trans coumaric acid	83.21	5.00	15.32
234, 280	289, 577.2	Proanthocyanidin dimer	Catechin	3441.81	10.99	633.46
238, 290sh, 326	192.9, 385	Feruroyl aldaric	Trans ferulic acid	401.30	2.72	73.86
238, 326	385	Feruroyl aldaric	Trans ferulic acid	828.33	13.20	152.45
236, 280, 314	289, 865	Proanthocyanidin trimer	Catechin	705.58	10.72	129.86
236, 322	208.8, 190.1, 415	Sinapic aldaric	Sinapic acid	940.32	2.26	173.07
236, 290, 320	191.9, 384.9	Feruroyl aldaric	Trans ferulic acid	69.53	7.86	12.80
234, 284, 324	289	Catechin	Catechin	719.23	5.26	132.38
232, 280, 520	447, 285	Cyanidin glucoside	Malvidin 6-glucoside	629.00	5.54	115.77
232, 278, 330, 426, 530	431, 269	Pelargonidin glucoside	Malvidin 6-glucoside	280.34	8.70	51.60
256, 354	301, 463	Quercetin glucoside	Quercetin	66.08	3.00	12.16
256, 294, 352	301, 549	Quercetin malonil glucoside	Quercetin	69.27	7.15	12.75

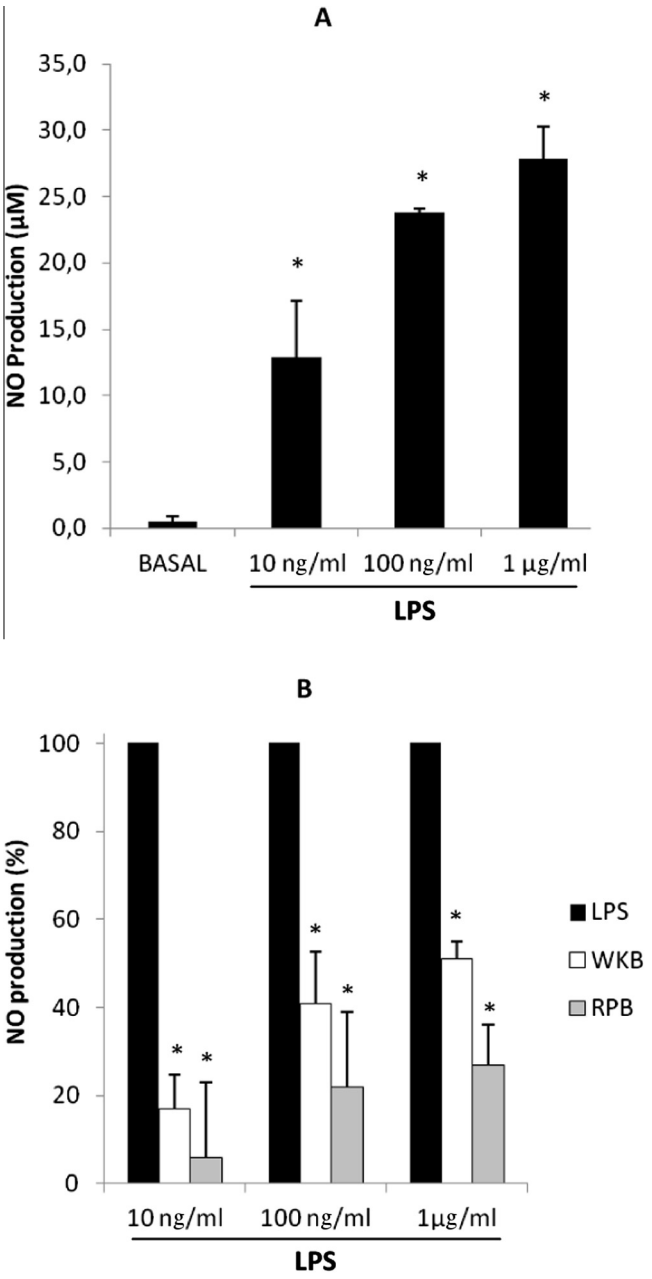

Fig. 1. Effects of methanolic bean extracts in NO production. Panel A: cells were stimulated with different concentrations of LPS (10 ng/ml, 100 ng/ml, 1 μg/ml) to induce NO production Panel B: cells were treated with 0.625 mg/ml of extracts: white kidney beans (WKB) or round purple beans (RPB) for 1 h prior to LPS stimulation. NO production is expressed as percentage of that of the group treated with LPS alone. Data are shown as mean ± SD of three different experiments $^{*}p < 0.05$.

compounds found were phenolic acids, especially hydroxycinnamic acids such as feruroyl aldaric and a derivative of the sinapic acid; most of them are aldaric derivatives. Apart from phenolic acids, flavanone has been detected in the extract. Also, flavonols, such as quercetin and derivatives, are present only in coloured beans. Anthocyanins, which are responsible for the purple colour of the bean, are detected in the RPB extract, too.

3.2. Effects of bean extracts on cell viability

The effect of different concentrations of methanolic legume extracts on cell viability was assessed by the MTT method. RPB extracts did not produce a significant effect on cell viability whereas the highest concentration (2.5 mg of extract/ml) of WKB extract induced a slight decrease on cell viability (data not shown). The lowest dose tested (0.625 mg of extract/ml) was selected for further experiments in order to evaluate their potential anti-inflammatory effect.

3.3. Inhibitory effect of methanolic bean extracts on NO production and iNOS mRNA expression in LPS-stimulated macrophages

To evaluate the effect of legume extracts on the macrophage inflammatory response induced by LPS, NO production was measured. LPS treatment induced high dose-dependent NO production (Fig. 1, panel A). Pretreatment with legume extracts reduced the NO production induced by all LPS concentrations, and this reduction was more evident when cells were stimulated with the lowest level of LPS (10 ng/ml). Moreover, RPB always exhibited a higher effect than WKB (Fig. 1, panel B).

The high levels of NO induced by stimulation with LPS are produced by the inducible isoform of the enzyme nitric oxide synthase (iNOS), which is synthesised by translation of the mRNA in the cytoplasma. To determine whether the inhibitory effect of extracts on NO production was due to inhibition of iNOS mRNA expression, RNA extraction and real time PCR were performed. As shown in Fig. 2, pretreatment with legume extracts reduced iNOS mRNA expression induced by different concentrations of LPS in a similar manner to the reduced NO production. Extracts from RPB were more effective than extracts from WKB.

3.4. Effect of methanolic bean extracts on cytokine gene expression induced by LPS

To investigate the effect of methanolic legume extracts on cytokine gene expression in LPS stimulated macrophages, mRNA expression was evaluated by real time PCR. LPS induces an elevated dose-dependent mRNA expression of the different genes studied (IL-1β, IL-6 and TNF-α). Pretreatment with legume extracts inhibited mRNA expression of all the genes tested when cells were

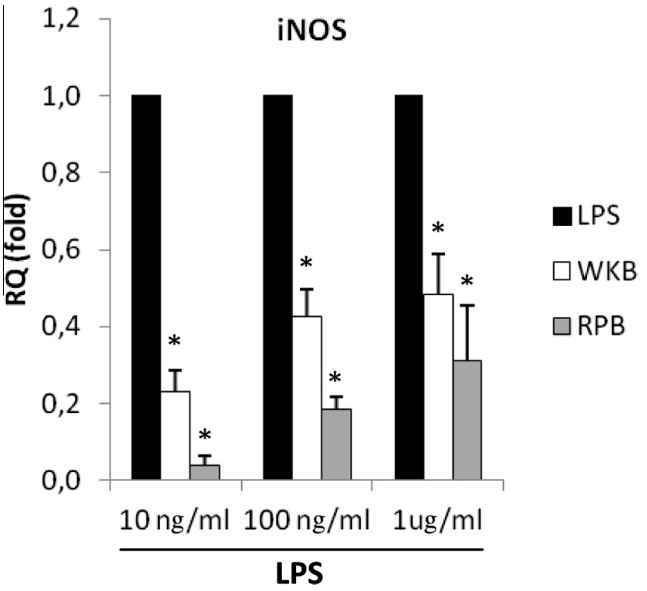

Fig. 2. Effect of methanolic bean extracts on mRNA iNOS expression. Macrophages were treated with 0.625 mg/ml of extract: white kidney beans (WKB) or round purple beans (RPB) during one hour before LPS stimulation with different concentrations. Results are expressed as fold of change (RQ) from reference treatment (LPS). Values show the mean ± SD of three different experiments $^{*}p < 0.05$.

stimulated with the lowest LPS dose (10 ng/ml). However when higher doses of LPS were used only IL-1β and IL-6 but not TNF-α mRNAs were reduced by legume extract pretreatment. In all cases RPB extracts showed a higher effect than the WKB ones (Fig. 3).

3.5. Effect of methanolic bean extracts on nuclear NF-κB p65 expression induced by LPS

In order to understand the mechanism underlying the inhibitory effect of the extracts on LPS induced inflammatory mediators, we studied the protein level of NF-κB p65 in the nuclear fraction of treated cells by Western blotting. The amount of NF-κB p65 in the nucleus of RAW 246.7 was dramatically increased upon stimulation with LPS. Treatment with WKB extracts induced a weak reduction of the expression levels of nuclear NF-κB p65, whereas treatment with RPB extracts produced a more evident inhibition (Fig. 4).

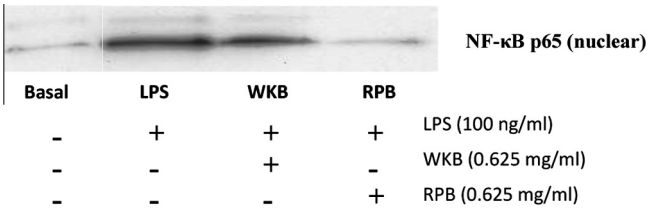

Fig. 4. Effect of methanolic bean extracts on expression of nuclear NF-κB p65. Cells were pretreated with 0.625 mg/ml of bean extracts: white kidney beans (WKB) or round purple beans (RPB) during 1 h and stimulated with LPS (100 ng/ml) for 30 min. Expression of nuclear NF-κB p65 was assessed by Western blot. The results are representative of three separate experiments.

4. Discussion

Polyphenols have been proven to act as antioxidants, protecting tissues from oxidative stress and pathologies associated with this condition. Besides their antioxidant properties, phenolic compounds have been shown to have anti-inflammatory activity by different mechanisms including modulation of the inflammatory cascade (García-Lafuente et al., 2009). The present work was aimed to investigate the antioxidant and anti-inflammatory properties of phenolic rich extracts, obtained from white and purple beans. The results demonstrated that both of them exhibited antioxidant and anti-inflammatory activities.

It has been reported that common beans have high contents of phenols compared to different fruits and vegetables, with pinto

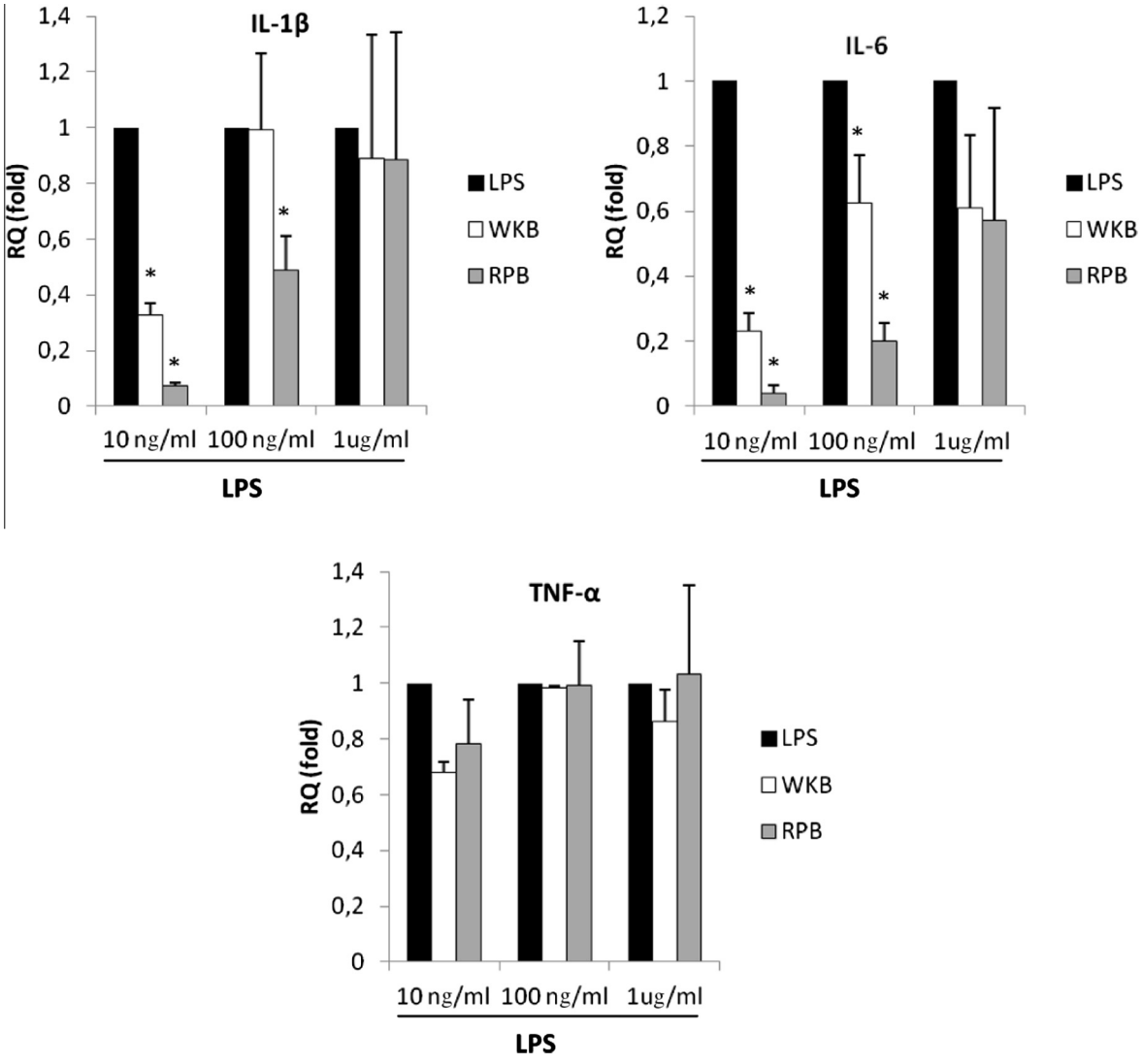

Fig. 3. Effect of methanolic bean extracts on mRNA expression of different proinflammatory genes. Cells were pretreated with 0.625 mg/ml of the extract: white kidney beans (WKB) or round purple beans (RPB) and then stimulated with different concentrations of LPS. Results are shown as fold of change (RQ) from reference treatment (LPS). Data represent the mean ± SD of three different experiments $^{*}p < 0.05$.

and kidney beans being a major source in particular (Espinosa-Alonso, Lygin, Widholm, Valverde, & Paredes-Lopez, 2006). In the present study the total phenolic content of extracts from white kidney beans and round purple beans was evaluated and the individual phenolic species were identified and quantified. The results obtained in both cases showed higher phenolic content in coloured beans than in white beans, and agree with those found in the literature for coloured and white beans (Anton, Ross, Beta, Gary Fulcher, & Arntfield, 2008; Heimler, Vignolini, Dini, & Romani, 2005; Ranilla, Genovese, & Lajolo, 2007; Xu, Yuan, & Chang, 2007).

The antioxidant and free radical scavenging power of phenolic compounds have been extensively studied over the last 10 years. Different studies have shown a correlation between phenolic content and antioxidant activity (Xu & Chang, 2008). In the present work the same results were obtained, the higher phenolic compounds content in bean extracts, the more antioxidant activity they exhibited. Extracts from red beans showed higher activity than those from white ones, in agreement with Madhujith et al. which demonstrated that beans, and especially those with coloured skins, possess strong antioxidant activity as measured by different model systems (Madhujith, Naczk, & Shahidi, 2004).

Macrophages play an important role in the cascade of events that produce inflammation. Stimulation of macrophages with LPS induces a high production of NO by the inducible enzyme iNOS (Boscá, Zeini, Través, & Hortelano, 2005; Gupta, Sundaram, Reuter, & Aggarwal, 2010). In the present work, bean extracts were able to reduce the production of NO in macrophages stimulated with LPS through the inhibition of iNOS mRNA expression. Besides NO, other mediators have been identified as important key factors in inflammation such as TNF-α or cytokines. There is a large body of evidence on the effect of phenolic compounds modulating inflammatory cascade at different levels, including the expression of inflammatory genes encoding interleukins (García-Lafuente et al., 2009). In our work, phenolic rich extracts from beans inhibited the expression of IL-1β, IL-6 and TNF-α genes of stimulated macrophages RAW 246.7, with coloured beans showing more activity than white beans.

Potential health benefits of common beans are attributed to the presence of phenolic compounds that possess antioxidant properties. The extent of anti-inflammatory activity may be associated with the antioxidant capacity and the phenolic content of the extract (Oomah, Corbé, & Balasubramanian, 2010). In our work, the studied extracts had a mixture of diverse phenolic compounds and the different compounds might contribute in different ways to the overall antioxidant and anti-inflammatory activities. The individual profile of phenolic compounds showed phenolic acids as the predominant components in white bean extracts, whereas coloured bean extracts presented mainly catechin derivatives, proanthocyanidins and catechin glucoside. These results are in accordance with bibliography (Aguilera, Estrella, Benitez, Esteban, & Martín-Cabrejas, 2011; López-Amorós, Hernández, & Estrella, 2006). Nevertheless, results obtained for coloured beans (RPB) presented higher concentrations of phenolic compounds, mainly catechin derivatives, procyanidins and catechin glucoside, which are only present in this kind of bean (Aguilera et al., 2011; Ranilla, Genovese, & Lajolo, 2009). Although most of these phenolic compounds are bioactive, the absence in WKB extracts of some potent active molecules such as catechin derivatives, proanthocyanidins, flavonols or anthocyanidins could explain the differences in activity found between both types of extract.

Epidemiological studies have indicated that populations that consume procyanidin rich foods have a lower incidence of inflammatory disease and diseases of multifactorial pathogenesis, including metabolic syndrome, atherosclerosis and cancer (Khan et al., 2010). Several *in vitro* and *in vivo* studies have shown that procyanidins can down regulate the transcription and secretion of proinflammatory cytokines, including interleukins IL-1β, IL-2, IL-6, TNF-α, and interferon-γ (Al-Hanbali et al., 2009; Terra et al., 2009).

NF-κB plays a critical role in the transcriptional regulation of a wide range of genes that are involved in inflammatory response and immunity. NF-κB proteins exist normally in the cytoplasm of inactive cells, but upon stimulation they become active and translocate into the nucleus, inducing transcription of diverse inflammatory genes. In this study we found that phenolic rich extracts from common beans reduced the expression of NF-κB p65 in the nucleus of LPS stimulated macrophage like cells. This effect was stronger for RPB than for WKB, suggesting that the studied extracts inhibited the expression of iNOS, IL-1β, IL-6 and TNF-α via inactivation of NF-κB pathway, although other points of regulation cannot be ignored. Ethanol extract derived from *Phaseolus angularis* was able to suppress NO and PGE_2 production at the transcriptional level, and this activity was linked to the suppression of some factors of different signalling pathways (Yu et al., 2001). Phenolic compounds from different origins have been proven to inactivate the NF-κB pathway (Denis et al., 2013; Park et al., 2012; Terra et al., 2007) so that in our work the inhibitory effect on the nuclear expression of the p65 subunit may be attributed to the phenolic composition of the extracts.

Although TNF-α is a very important mediator involved in the inflammatory response in our work, the treatment with bean extracts was not able to reduce gene expression in response to high dose of LPS. We found similar results using mushroom extracts to reduce inflammatory response to LPS; phenolic rich extracts from some edible mushrooms species were able to reduce the production and expression of NO, IL-1β, and IL-6 but not TNF-α (Moro et al., 2012). Regulation of the expression of different inflammatory genes is controlled by different signal transduction pathways such as NF-κB and mitogen-activated protein kinase (MAPK). The inhibition of NF-κB produced by the extracts was not enough to reduce the TNF-α mRNA expression, suggesting that expression of different cytokines are regulated by different pathways.

5. Conclusions

Our work demonstrates that common beans exhibit both antioxidant and anti-inflammatory activities. Round purple bean extracts present higher phenolic content than white bean extracts and a different component profile related to differences in activity. Higher phenolic content was associated with higher antioxidant and anti-inflammatory activities. These data suggest that common beans, and in particular coloured beans, may be used as a source of anti-inflammatory compounds.

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