Proximate Composition, Polyphenol Content and Anti-inflammatory Properties of White and Pigmented Italian Rice Varieties

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Abstract Rice (*Oryza sativa L.*) is a major staple food for the majority of the world population. Grown and harvested as paddy, it is present on our tables as white rice (milled), brown rice (dehusked) or, after being subjected to thermal processes, as parboiled rice. Recently, pigmented varieties are also appreciated (red and black rice). In this study, we evaluated proximate composition, polyphenol content and anti-inflammatory properties of Italian pigmented rice varieties and bran as well as white rice in different processed forms (milled, parboiled and integral), in order to assess the nutritional and antioxidant properties of Italian rice varieties and their capacity to reduce inflammation. Our results showed that pigmented rice varieties displayed high antioxidant capacity along with the highest flavonoid and polyphenol content. Anthocyanins were mainly present in black rice, whereas alkylresorcinols were found only in red rice. Rice bran proved to be particularly rich in carotenoids. The evaluation of the anti-inflammatory properties using lipopolysaccharide (LPS)-induced mouse RAW 264.7 macrophages pre-treated with extracts obtained from integral, black, red rice and bran showed that the expression of inducible Nitric Oxide Synthase (iNOS) was specifically reduced at both transcript and protein level by extracts, indicating the capacity of rice extracts to modulate the inflammatory response.

Keywords Rice, Nutritional Properties, Antioxidant Capacity, Anti-inflammatory Properties

1. Introduction

Rice is the third most important cereal food in the world and the staple food in many Asian countries. The most common rice consumed by humans is white rice (about 85%), but an increasing interest emerged around pigmented rice varieties, such as black, red and dark purple rice, due to the considerable amount of polyphenols present in the external layers of rice kernels. Black and purple rice mainly contain anthocyanins, a specific class of flavonoids conferring red to purple color [1], whereas pro-anthocyanidins are the main polyphenols in red rice [2]. Several beneficial properties, such as antioxidant, antitumoral, anti-atherogenic, anti-obesity, hypoglycemic and antiallergic effect have been reported for pigmented rice, especially black and dark purple rice [3]. Pigmented rice is popular and consumed mostly in Asian countries and the properties of many Asian rice varieties have been characterised so far [3]. On the other hand, the knowledge around the nutritional and health-promoting properties of Italian white and pigmented rice varieties is currently very limited [4, 5].

Rice is mainly consumed as milled rice, obtained by removing the hull and bran layer from the rice kernel. After removal of husk, rice is referred to as brown rice, which consists of bran (6-7% by weight), endosperm (about 90%) and embryo (2-3%) [6]. White rice, also referred to as milled or polished rice, is obtained by removing 8-10% of external layers (mainly bran) from brown rice [7]. Although the polishing process provides benefits to the physical and sensorial properties of rice, it depletes rice of most nutrients and phytochemicals present in the kernels [8]. Rice bran is the main by-product derived from rice milling and is rich in proteins, fibers, vitamins, fats and polyphenolic compounds, which are mainly located in the external layers of the rice kernel [9]. Rice bran has recently received considerable attention due to its beneficial health effects against diabetes, cancer and heart disease [3]. Its use as largely available and inexpensive source of natural antioxidants and bioactive peptides with health-promoting properties has been recently evaluated [10, 11].

Parboiling of rice is an effective processing technique to

increase storage stability of rice with minimal changes in nutritional properties. It occurs before dehusking and consists of soaking, pressure steaming and drying, after which rice undergoes conventional processing. During parboiling, the transfer of bran components to the inner layers of rice kernels, the inactivation of lipase and starch retrogradation have been widely documented [12]. Interestingly, parboiling of pigmented rice has been recently shown to allow the partial preservation of free phenolics, which would be otherwise lost in polishing process of rice [8].

Rice has been considered a source of carbohydrate with low to moderate dietary fiber content, based on the analysis of total dietary fiber and resistant starch. The amount of resistant starch varies in different rice cultivars, depending on the amylose content, and it can be increased by the parboiling process [13]. Due to its chemical nature and low digestibility, resistant starch is considered a type of dietary fiber. Resistant starch is the fraction of starch, resistant to enzyme hydrolysis, entering the large intestine along with dietary fibers. As part of dietary fiber, resistant starch is fermented by the gut microbiota to produce short chain fatty acids that signal satiety and impact insulin signalling [14]. Thus, resistant starch from rice has the potential to improve human health; nevertheless its content in commonly consumed rice cultivars or pigmented rice varieties needs further characterization.

Inflammation represents the main response against injury and it is normally characterised by an early acute phase, followed by the resolution of inflammation [15]. This process involves various cell types, including leukocytes that are recruited to the site of injury through chemotaxis. Failure in promoting resolution results in the establishment of a chronic state of inflammation, which represents a risk factor of various types of diseases, including cancer, diabetes, cardiovascular and autoimmune diseases [15]. Therefore, dietary strategies to reduce acute inflammation and prevent its degeneration towards the chronic state are attracting great interest.

The present study aimed at characterizing the proximate composition, polyphenol content and anti-inflammatory properties of Italian pigmented rice varieties and bran as well as white rice in different processed forms (milled, parboiled and integral), in order to assess the nutritional and antioxidant properties of Italian rice varieties and their capacity to reduce inflammation.

2. Materials and Methods

2.1. Materials

The current Italian legislation provides that all rice varieties suitable for trade are listed in an annual ministerial decree (currently valid: DM 30 September 2016, No 275, GU 24/11/2016), divided into product groups. Based on this classification, the following Italian rice varieties from Ente Nazionale Risi germplasm were analyzed: a typical white variety suitable for cooking the risotto in three commercially available forms (milled rice, parboiled rice and integral rice), a black variety, a red variety and a mixture of rice bran kindly provided by Euricom Spa (Vercelli, Italy).

2.2. Proximate Analysis

Protein content was calculated from nitrogen content assessed by the Kjeldahl method using a 5.95 conversion factor. A test portion of milled rice (1g) was digested by sulfuric acid 18 mol/L (20 mL) in the presence of a catalyst. The reaction products was made alkaline, and then distilled. The liberated ammonia was collected in a boric acid solution, which was titrated with a sulfuric acid solution, to determine the nitrogen content and calculate the crude protein content according to ISO 20483:2006 [16]. For crude fat, the Soxhlet extraction method with petroleum ether as solvent was used, according to AACC Method 30-25.01 [17]. Briefly, 5g of dried (in a vacuum oven: 100 mm Hg; 5 h) grinded rice were extracted with 100mL of petroleum ether (30-60°C) for 2 h. Moisture content was determined by using the standard methods of analysis ISO 712:2009 [16] and expressed as percentage. In particular, 5 g of grinded rice were dried in a Memmert UFE 400 oven for 2hs. Ash content was determined according to the AACC method 08-01 [17]. After determining the amount of dietary fiber and subtracting it from total carbohydrate, available carbohydrates were estimated by difference as follows: 100 - weight in grams [protein + fat + moisture + ash + dietary fiber] in 100 g of rice sample. Dietary fiber was determined according to method AOAC 985.29 [18].

2.3. Determination of Total and Resistant Starch and Amylose Content

The amount of total and resistant starch were determined following the AACC Method 32-40.01 using the K-RSTAR kit (Megazyme, Wicklow, Ireland), according to manufacturer's instructions. Rice flour (100mg) was incubated with 4mL pancreatic α-amylase (10 mg/mL) containing amyloglucosidase (3U/mL) in a shaking water bath at 37°C for 16 h. Then, ethanol (4mL, 99% v/v) was added and samples were centrifuged at 2000g for 10 mins. Pellets were resuspended with 2mL ethanol (50% v/v) and, after adding 6mL ethanol (50% v/v), samples were centrifuged at 2000g for 10 mins. Pellets were then resuspended and centrifuged with the same procedure. To measure the resistant starch content, pellets resuspended by stirring with 2 mL potassium hydroxide (2 M) and incubated in an ice water-bath for 20 min. After adding 8 mL of sodium acetate buffer (1.2 M, pH 3.8) and 0.1 mL amyloglucosidase (3300 U/mL), samples were incubated in a water bath at 50°C for 30 min and then centrifuged at 1500 g for 10 min. Aliquots of supernatants (0.1 mL) were incubated into glass

test tubes with glucose oxidase/peroxidase (GOPOD) reagent in a water bath at 50°C for 20 min. Absorbance was immediately measured at 510 nm compared to the following reagent blank (0.1 mL of 100 mM sodium acetate buffer pH 4.5 and 3 mL of GOPOD reagent). To measure the digestible starch, the supernatants obtained by the three initial centrifugations were combined, the volume adjusted to 100 mL with 100 mM sodium acetate buffer pH 4.5 and a 0.1 mL aliquot was incubated with 10 μL of amyloglucosidase solution (300 U/mL) for 20 min at 50°C. After adding 3 mL of GOPOD reagent, samples were incubated at 50°C for 20 min. The absorbance was measured at 510 nm against a reagent blank.

The resistant and non-resistant starch content was calculated using the Megazyme Mega-Calc^{TM} program (Megazyme, Wicklow, Ireland). The total starch content is the sum of resistant and non-resistant starch content. The starch content was expressed as g/100g of rice flour.

For the determination of amylose content, rice was ground to a very fine flour (Retsch ZM200 grinding system and sieving with a 150 μm Endecotts sieve) to break up the endosperm structure in order to aid complete dispersion and gelatinization; the flour was then defatted. A test portion was dispersed in a sodium hydroxide solution, to an aliquot portion of which an iodine solution was added. The absorbance, at 720 nm, of the color complex formed was then determined using a UV-VIS spectrophotometer (Lambda 25, Perkin Elmer). The amylose mass fraction of the sample was then read with a calibration graph, which was prepared using mixtures of potato amylose and amylopectin to make allowance for the effect of amylopectin on the colour of the amylose–iodine complex of the test solution, according to the ISO 6647-1:2007 method [16].

2.4. Determination of Antioxidant Capacity and Total Polyphenol, Flavonoid and Anthocyanin Content

Methanolic extracts were obtained from 0.5 g of rice flours with 20 mL of methanol-1% HCl and used according to the procedures described below.

The antioxidant capacity was measured by determining the ability of methanolic extracts to scavenge 2,2 azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The ABTS radical scavenging activity was measured by using the TEAC (Trolox Equivalent Antioxidant Capacity) method as previously described [19] and expressed as μmol of Trolox equivalents (TE) per 100 g of rice flour.

The total phenolic content was determined using the Folin–Ciocalteau method. Methanolic extracts were mixed with Folin-Ciocalteau's phenol reagent 0.2 N (in a ratio of 1:5). Sodium carbonate (7.5 %) was added into the mixtures (in a ratio of 1.5:1) and incubated for 2 h. The reaction mixture was then read at 760 nm against a standard curve of gallic acid and total polyphenol content was reported as mg of gallic acid (GA) equivalents per 100 g of rice flour.

To measure flavonoid content, methanolic extracts were incubated with 2% $AlCl_3$ for 25 min and then read at 510 nm against a standard curve of quercetin. The flavonoid content was expressed as mg quercetin (Qc) equivalent/100g of rice flour. With this method it is possible to determine the total content of flavonoids, including chalcones, flavones, flavonols, and anthocyanins.

The total anthocyanin content was measured with the pH-differential method. Briefly, 1 mL of the methanolic extracts was treated separately with 4 mL of pH 1.0 buffer (25 mM KCl pH 1) or 4 mL of pH 4.5 buffer (400 mM sodium acetate pH 4.5). The samples were thoroughly mixed and the absorbance was measured at 520 nm and 700 nm. The total anthocyanin content was calculated according to the equation:

Amount [mg g^{-1}] = ΔA × 449.2/26900 × dilution factor x final volume (mL)/sample weight (g) where ΔA: (A_{510}-A_{700}) pH1- (A_{510}-A_{700}) pH4.5 . The value 449.2 represents the molecular mass of cyanidin 3-glucoside, whereas 26900 is its molar absorbance. Each sample was analyzed in triplicate and the anthocyanin content expressed as mg cyanidin 3-glucoside (C3G) equivalents per 100 g of rice flour.

2.5. Determination of Alkylresorcinols

To determine the alkylrercinol content, 0.2 g of rice flour was extracted with 8 mL of acetone, after 48 h of shaking the samples were centrifuged and the supernatant dried under vacuum and redissolved in 200 μL of methanol. For the colorimetric reaction, 10 μL of each sample were treated with 2 mL of Fast Blue Zn Salt 0.05% freshly diluted with 5 volumes of methanol. After incubation of 1 h in the dark, the samples were read at 520 nm. The alkylresorcinol content was calculated using a calibration curve made with 5-pentadecylresorcynol (C21) as standard and expressed as mg/100g of rice flour.

2.6. Determination of Carotenoid Content

Carotenoids were extracted as previously reported [20]. Briefly, 6 mL of methanol, acetone and hexane (1:1:1, v/v) were added to a glass tube containing a sample of 0.5 g of rice flour and shaken horizontally for 30 min. After centrifugation, 1 mL of the non-polar phase containing carotenoids was filtrated through a 0.22 μm filter and analyzed spectrophotometrically at 448 nm. The carotenoid content was calculated from the calibration curve of lutein as standard and expressed as μg lutein equivalents per 100 g of rice flour.

2.7. Cell Culture

Mouse RAW 264.7 macrophages were purchased from Sigma Aldrich (91062702-1VL) and cultured in DMEM (Sigma), supplemented with 10% fetal bovine serum (Sigma), 100 μg/mL penicillin/streptomycin (Sigma) and 2 mM L-glutamine (Sigma). Cells were maintained at 37°C in 5%

CO_2 atmosphere. The cells were in the exponential phase of growth before exposure to extracts in all experiments.

Rice extracts were prepared from 12g of each rice flour (*i.e.* integral, red, black rice and bran) with 20 mL/g of n-hexane and the mixture was shaken at 250 rpm for 30 min at room temperature. After centrifuging at 10000 g for 10 min, the residue was dried at room temperature in a fume hood and then extracted twice with methanol-1% HCl. After shaking at 250 rpm for 45 min per extraction, the mixture was centrifuged at 10000 g for 15 min. The supernatants of each methanol extraction were combined, concentrated at equal volumes using a vacuum refrigerated centrifuge and stored at −20 °C for further analyses.

Prior to the experiments, RAW 264.7 cells were plated at a density of 35000 cells/well in 96-well plates and cultured for 48 h. The cells were treated for 48 h with different concentrations of methanol extracts (0, 5, 25, 125, 250 μM). Cell viability was determined using 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) test. Briefly, 10% of MTT solution per volume was added to each well and incubated for additional 4 h. Formazan crystals were dissolved in acidified isopropanol (isopropanol, 0.1N HCl, 0.1% Tween). The optical density was measured at 570 nm wavelength, by using a microplate reader (Tecan Infinite F200PRO).

RAW 264.7 macrophages were plated at a density of 1×10^6 cells/well in 6-well plates and grown for 24 h. The cells were pre-treated with the chosen dosage of extracts (125 μM) for 16 h and then 100 ng/mL LPS (Sigma L4391) was added for additional 6 h for RNA extraction or 24 h for supernatants and protein analysis.

2.8. RNA Extraction and qRT-PCR Analysis

Table 1. Primer sets used for qRT-PCR analysis

Gene	Primer name	Sequences (5′→3′)
TNF-α	TNFa-F	GGAGTTTGAGTCTGCAGAGTTCC
	TNFa-R	GGGGAAGGCATTAGAAACAGTCC
IL-1β	mIL1bfor	GGTGTGTGACGTTCCCATTA
	mIL1brev	ATTGAGGTGGAGAGCTTTCAG
IL-6	mIL6for	CAGAGTCCTTCAGAGAGATACA
	mIL6rev	CCTTCTGTGACTCCAGCTTATC
iNOS	miNOSfor	TGGTGTCTGCAGCACTTGGA
	miNOSrev	TTGAGCCCTTTGTGCTGGGA
COX-2	mCOX2for	CGAGTCGTTCTGCCAATAGAA
	mCOX2rev	CCTGGTCGGTTTGATGTTACT
GAPDH	GAPDH-Fw	ATGTGTCCGTCGTGGATCTG
	GAPDH-Rv	GGTGGAAGAGTGGGAGTTGC

Total RNA was extracted using Direct-zol^TM RNA Miniprep kit (Zymo Research). Synthesis of cDNA was obtained using the RT SuperscriptTM II (Invitrogen, Carlsbad, CA). Transcript analysis was performed using SOS Fast EVA-Green Supermix (BioRad Laboratories, Hercules, CA) on a Cfx96™BioRad Real Time system. Values were normalized on transcript level of the *GAPDH* housekeeping gene and expressed as Fold Change (FC) relative to untreated control sample. Primer sequences are indicated in Table 1.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants were analyzed for mIL6, mIL1β, mTNFα and mIL10 using Ready SET-Go! ELISA kit (Affymetrix) according to the manufacturer's instructions. The optical density (O.D.) of each well was analyzed at 450 nm by a Tecan Microplate Reader (Tecan).

2.10. Western Blot Analysis

Total proteins were extracted using RIPA buffer (10mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1mM PMSF), lysates were centrifuged and the supernatants were collected. Equal amounts of proteins (30 μg) underwent SDS–PAGE and were transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies against Anti-iNOS (Cayman chemical, ab160862) or α-Tubulin (Sigma, T6074) and probed with horseradish peroxidase-conjugated secondary antibodies. Bands were detected using Pierce™ ECL Western Blotting Substrate (ThermoFisher Scientific). All the antibodies were applied at 1:2000 dilutions.

2.11. Statistical Analysis

Analytical determinations for the samples were performed in triplicate on at least three biological samples. All values were expressed as means ± SEM, except where specified. Data analysis was carried out by one way ANOVA and Tukey's means comparison test with GraphPad Prism version 6.0 (GraphPad Software). Differences were considered significant when $p < 0.05$.

3. Results and Discussion

3.1. Proximate Composition

The proximate compositions of white rice in three processed forms (milled, integral, and parboiled), red and black rice as well as bran are presented in Table 2. The moisture content of all three Italian varieties, including different processed forms of white rice, and bran were lower than 14%, which is considered safe for storing grains to avoid damage and deterioration of seed quality [9].

Table 2.   Proximate composition of milled, parboiled and integral rice, red and black rice, bran

Component (%)	Milled	Parboiled	Integral	Red	Black	Bran
Moisture	10.87±0.15	11.94±0.32	10.72±0.24	10.78±0.19	10.59±0.28	9.11±0.32
Carbohydrates	81.24±7.90	79.28±7.48	75.04±6.80	71.20±6.85[c]	73.39±7.12[c,d]	27.45±2.47[a]
Proteins	5.78±0,52	5.57±0,53	6.24±0,58	7.88±0,69	7.37±0,80	12.4±0.95[a]
Lipids	0.93±0.05	1.11±0.04	2.91±0.50	4.32±0.54	3.16±0.14	19.08±0.90[a]
Ash	0.29±0.06	0.67±0.06	1.25±0.09	1.42±0.09	1.58±0.08	8.86±0.89[b]
Dietary fiber	0.89±0.08	1.43±0.19	3.84±0.34	4.40±0.39	3.91±0.35	23.10±2.07[a]

Means of three determinations with standard deviations. Bran values were significantly different from all other rice samples at p<0,001 (a) or p<0,05 (b). Red and black rice values were significantly different from milled rice (c) or from parboiled rice (d) at p<0,05.

No significant differences were observed in carbohydrate content of milled, parboiled and integral rice ranging from 75.04% to 81.24%, whereas in red and black rice carbohydrates were significantly lower than milled and parboiled rice (Table 2). Bran had a significantly lower content of carbohydrate, whereas lipid, ash and dietary fiber content was significantly higher compared to all other rice (Table 2). This was expected, since bran is mainly constituted of external layers of rice kernels, where proteins, lipids and dietary fiber are mostly concentrated [9].

Despite differences among the other rice samples were not statistically significant, content of lipids, ash, proteins and dietary fibers were lower in milled and parboiled rice, whereas integral, red and black rice showed higher content of such components, as a consequence of a lower degree of milling (Table 2). In particular, lipids can contribute to the quality of rice, influencing the nutritional and sensory aspect, although not abundant such as carbohydrates and proteins [21]. Lipid content in milled and parboiled rice was 0.93-1.11%, whereas in integral, red and black rice it ranged from 2.91 to 4.32% (Table 2). Accordingly, the total concentration of lipids in the caryopsis of unmilled rice has been reported between 2 and 4%, while concentration in the grain milled rice amounts to 0.3 to 0.6% [22]. Similarly, protein content in milled and parboiled rice was lower (5.57-5.78%) compared to integral, red and black rice (6.24-7.88%) (Table 2). Proteins play an important role in cooked rice texture, because they form a complex with starch that impairs starch granule swelling [21]. High protein rice is less sticky and has a harder texture, but rice obtained from a higher degree of milling has a comparatively better sensory quality [23].

The total dietary fiber content in milled and parboiled rice was found to range between 0.9-1.4%, whereas it was higher in integral, red and black rice (3.8-4.4%), in which more external layers of rice kernels are present (Table 2). Dietary fiber has been correlated to a reduced risk of type-2 diabetes and cardiovascular diseases. It can reduce appetite and contribute to weight loss, since it is not digested in the small intestine, but is fermented by microbiota in the colon, where it produces short-chain fatty acids, such as acetate, butyrate and propionate, which are important for satiety signalling [14].

3.2. Resistant Starch, Digestible Starch and Amylose Content

The total starch content was 62-69% in milled, parboiled and integral rice, whereas red and black rice contained a significantly lower content of starch (about 60%) compared to milled rice, probably due to a lower degree of milling and the consequent presence of external seed integuments (Figure 1A). Consistently, rice bran has a very low total starch content compared to milled rice, being mainly constituted of external layers of rice kernels (Figure 1A).

Most of starch present in rice consists of digestible starch (Figure 1B), but in parboiled rice resistant starch is significantly higher compared to milled rice and, despite not significant, a similar trend was observed for integral rice (Figure 1C). Foods with higher levels of resistant starch have been reported to reduce glucose and insulin responses and confer enhanced satiety as well as to reduce potential risk factors for type 2 diabetes and the metabolic syndrome [24].

Amylose content is considered the single most important determinant for predicting the eating quality of rice [23]. Amylose content is directly related to water absorption, volume expansion, fluffiness and separability of cooked rice. It is inversely related to cohesiveness, tenderness, glossiness and cooked grain stickiness, whereas it is positively correlated with cooked grain [23]. Based on amylose content, rice can be classified in 5 groups. Most Italian rice varieties are classified within Group III with a total amylose content ranging between 12 and 15 g/100g, except some of them which are classified with Group IV with an amylose content of 25-27 g/100g [22]. Total amylose content of white rice in the three processed forms (milled, parboiled, integral), of red and black rice as well as bran is presented in Figure 1D. The total amylose content in milled rice was found to be approximately 17.4%, thus classified under rice quality Group III [22]. Despite red and black rice contained a significantly lower amylose content compared to milled rice (approximately 9.9 and 10.5 g/100g, respectively), they can also be classified within Group III (Figure 1D). Again, rice bran has only traces of amylose content compared to milled rice, being mainly constituted of external layers of rice kernels (Figure 1D).
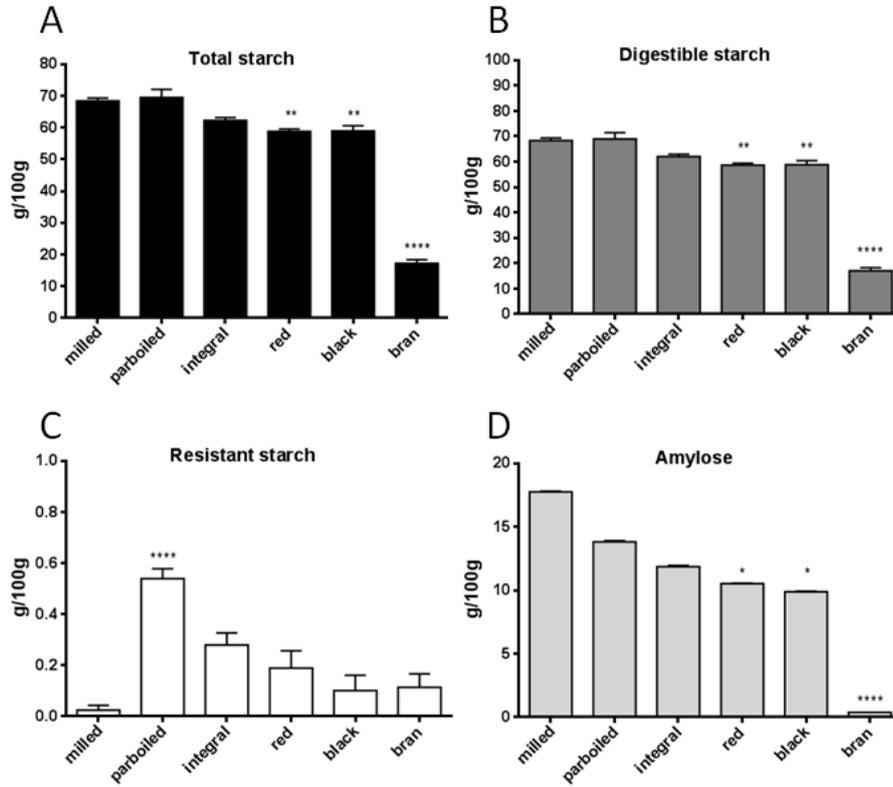
Figure 1. Determination of starch composition. Total, digestible and resistant starch (A-C) and amylose content (D) were determined in white rice in three different processed forms (milled, parboiled, integral) and in red, black rice and bran and expressed as g/100g ****p<0.0001; ***p<0.001; **p<0.01 indicate significant differences (one-way ANOVA) *versus* milled rice.
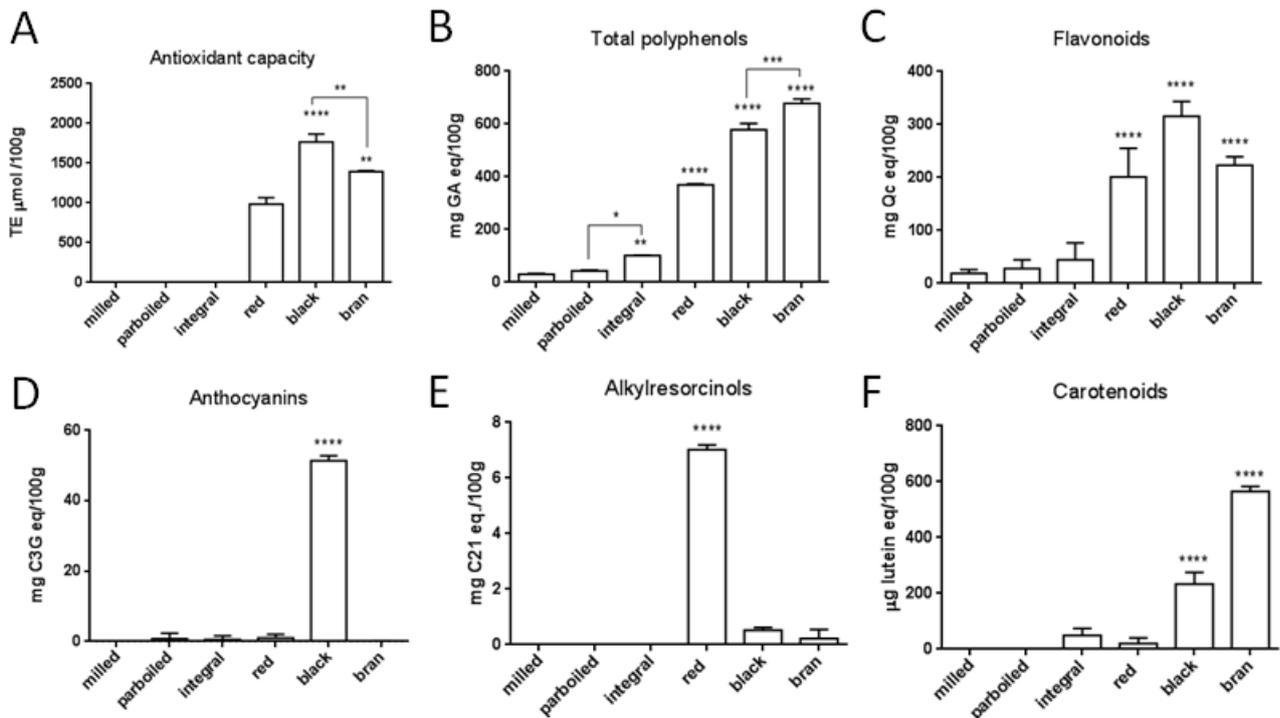
Figure 2. Determination of (A) antioxidant capacity expressed as μmol of trolox equivalents (TE) per 100 g, (B) total polyphenols expressed as mg of gallic acid (GA) equivalents per 100 g, (C) flavonoids expressed as mg quercetin (Qc) equivalents per 100 g, (D) anthocyanins expressed as mg cyanidin 3-glucoside (C3G) equivalents per 100 g, (E) alkylresorcinols expressed as 5-pentadecyl resorcynol (C21) equivalents mg per 100 g and (F) carotenoids expressed as g lutein equivalents per 100 g. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05 indicate significant differences (one-way ANOVA) *versus* milled rice.

3.3. Total Polyphenol Content and Antioxidant Activity

Milled, parboiled and integral rice did not show antioxidant activity, consistent with the almost complete removal of external integuments, whereas the two pigmented rice cultivars, especially black rice, and bran showed a significantly high antioxidant capacity (Figure 2A). Pigmented rice cultivars and bran showed higher total polyphenol content (372.7–678.0 mg gallic acid (GA) equivalents /100g) than the remaining non-pigmented rice (30.5–101.3 mg GA equivalents /100g). Nonetheless, integral rice showed significantly higher polyphenol content compared to milled rice (Figure 2B). Higher levels of antioxidant activity of red, black rice and bran (984.20–1768 TE μmol/100g) were strongly correlated with total polyphenol content (Figure 2A-B).

3.4. Flavonoids, Anthocyanins, Alkylresorcinols and Carotenoids

The analysis of the different classes of polyphenols associated with rice cultivars and bran showed that in integral rice polyphenols were mainly represented by flavonoids (Figure 2C), a small fraction of which consisted of anthocyanins (Figure 2D). Similarly, bran mainly contained flavonoids (Figure 2C) and a small quantity of anthocyanins and alkylresorcinols (Figure 2D,E). Concerning black rice, polyphenols were mainly represented by anthocyanins and to a smaller extent alkylresorcinols (Figure 2D,E), whereas in red rice a high level of alkylresorcinols was detected (Figure 2E). Significant differences in the amount of anthocyanins in black and red rice cultivars of different origins have been previously reported [1, 4, 25]. The data obtained from our analysis is consistent with these studies, since the anthocyanin content of black rice was 51.4 mg/100g, whereas that of red rice was only 0.98 mg/100g. It is known that pigmentation in red rice is mainly due to pro-anthocyanidins [2], but the presence of alkylresorcinols in pigmented rice cultivars has not been previously ascertained. High levels of alkylresorcinols were reported in wheat, rye and triticale and low amounts in barley, millet and maize. Although alkylresorcinols were found in rice seedlings, they were absent in kernels of rice [26]. Our data showed that alkylresorcinols were mainly present in red rice, but to a very low amount also in black rice and bran (Figure 2E). Alkylresorcinols have anticancer, antimicrobial and antioxidant activity *in vitro* and are mainly found in bran layers of cereal grains, which mean that they are lost in refined cereals [27]. Therefore, red rice may represent an additional source of these beneficial compounds, with an amount of alkylresorcinols (7 mg/100g) similar to barley (4.2-5.1 mg/100g) [26]. Interestingly, alkylresorcinols have been proposed as biomarkers of whole cereal grain intake when measured in plasma, in order to understand the preventive effect of whole grain consumption against chronic diseases [27].

The carotenoid content varied substantially among the different rice samples. Carotenoids were not detected in milled and parboiled rice, whereas the levels of carotenoids were 48.98, 20.59 and 232.8 μg/100g in integral, red and black rice, respectively (Figure 2F). The total amounts in red and black rice were significantly lower from those reported in Korean or Camargue pigmented rice [25, 28], but the relative amount between red and black rice was similar, since the carotenoid content was 9-11 fold higher in black rice compared to red rice cultivars of similar origin. The carotenoid content found in bran was 11.6-fold higher than in integral rice and 2.4-fold higher than in black rice (Figure 2F), consistent with previous studies indicating that carotenoids are mainly located in rice bran [29].
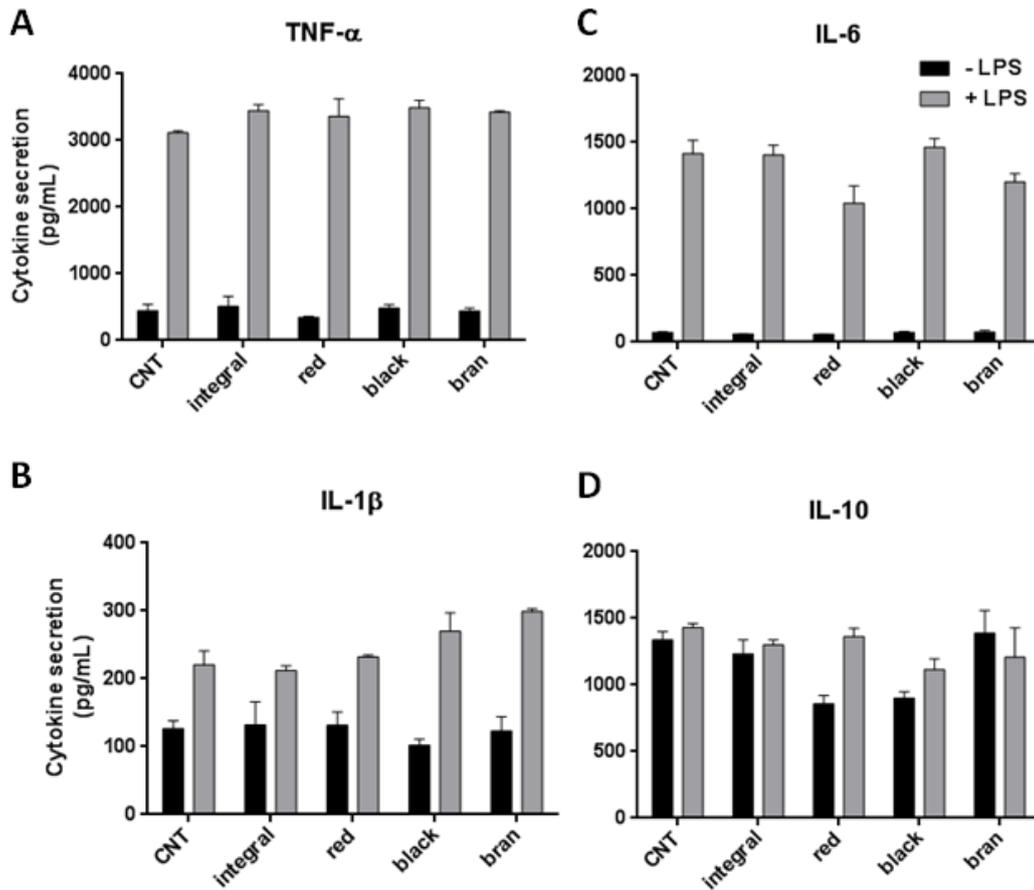
3.5. Anti-inflammatory Effect of Rice Extracts on LPS-induced RAW 264.7 Macrophage Cells

In order to evaluate the anti-inflammatory effect of integral, red, black rice and bran, methanol extracts were prepared and a non-cytotoxic dose (125 μM) determined by MTT assay, which showed no growth effects in RAW macrophage cells (data not shown), was used in later experiments.

Pathogen-induced acute inflammation induces activation of macrophages, resulting in production of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 acting as inducers of acute phase reactions, but also an increase of nitric oxide (NO) production, regulated by inducible nitric oxide synthase (iNOS), and of COX-2 expression and its enzymatic product prostaglandin E2 (PGE2) [15]. Therefore, the expression of pro-inflammatory genes (*i.e. TNF-α, IL-1β, IL-6, iNOS* and *COX-2*) was analyzed by qRT-PCR in LPS treated RAW 264.7 murine cell line. Cells were pre-incubated for 16 h with methanolic extracts from integral, red, black rice and bran and then treated with LPS for additional 6 h. Transcript levels of all pro-inflammatory genes were strongly increased upon stimulation with LPS (Figure 3, CNT+LPS). Pre-treatment with rice extracts did not affect *TNF-α, IL-6* and *COX-2* genes (Figure 3A, C, E), whereas *IL-1β* transcript level increased with respect to CNT+LPS (Figure 3B). On the contrary, pre-treatment with rice extracts reduced the expression of *iNOS* compared to CNT+LPS (Figure 3D).
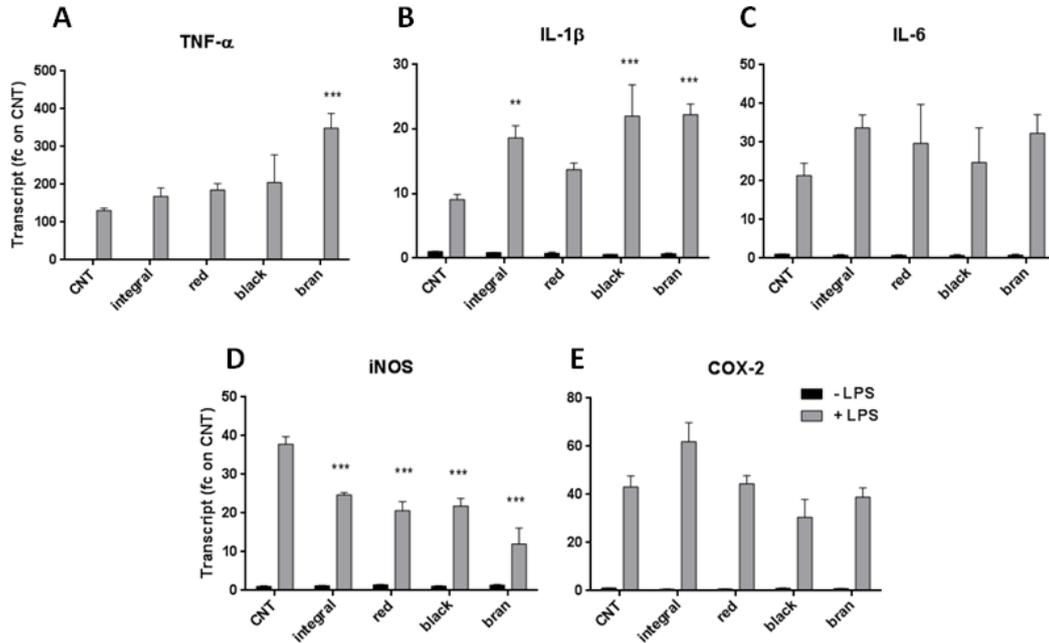
Figure 3. Expression levels of *TNF-α, IL-1β, IL-6, iNOS* and *COX-2* transcripts in mouse RAW 264.7 macrophages pre-treated for 16 h with 125 μM of extracts obtained from integral, red, black rice and bran followed by exposure to 100 ng/mL LPS for 6 h. Each transcript was expressed as fold change compared to CNT-LPS. ***p<0.001; **p<0.01 indicate significant differences (one-way ANOVA) *versus* CNT+LPS.
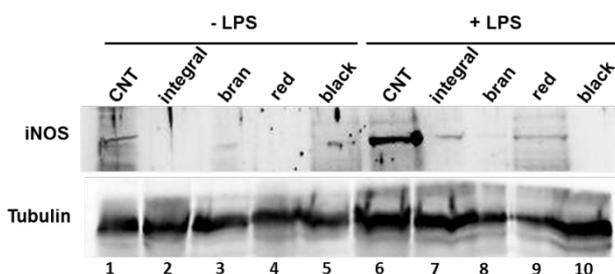

Figure 4. Secretion of pro-inflammatory and anti-inflammatory cytokines. Mouse RAW 264.7 macrophages pre-treated for 16 h with 125 μM of extracts obtained from integral, red, black rice and bran followed by exposure to 100 ng/mL LPS for additional 24 h. No significant differences were observed (one-way ANOVA) *versus* CNT +LPS.

3.6. Effect of Rice Extracts on Pro-inflammatory Cytokine Secretion and iNOs Protein Levels in LPS-induced RAW 264.7 Macrophage Cells

We then analyzed the secretion of pro-inflammatory cytokines by ELISA to confirm the effects observed at the transcriptional level. The TNF-α, IL-1β and IL-6 secretions were all induced by LPS (Figure 4A-C) and remained unaffected by the pre-treatment with rice extracts, confirming that rice extracts do not prevent the release of pro-inflammatory mediators. In order to evaluate their potential effect on anti-inflammatory cytokines, the secretion of IL-10 by macrophages was evaluated. As shown in Figure 4D, IL-10 secretion was not induced by LPS and pre-treatment of cells with rice extracts did not increase its expression compared to CNT+LPS, indicating that rice extracts have no effect on IL-10.

We then verified whether the reduced transcript level of the *iNOS* gene correlated with a decrease of iNOS protein (Figure 5). The expression of iNOS protein was up-regulated in LPS-induced RAW macrophage cells. However, iNOS protein level was significantly decreased when the cells were pre-treated with rice extracts (Figure 5). Since no effect was observed on cytokines production, these results suggest that rice extracts exert their anti-inflammatory action by inhibiting iNOS, an inflammatory mediator regulating NO production upon infection. Similar findings of a flavonoid rich-extract reducing the expression of iNOS protein and corresponding transcript have been reported with extracts from black rice bran fraction or *Ginko biloba in vitro* [30, 31]. A modest decrease of iNOS protein expression has been also observed *in vivo* in aortic tissue of ApoE mice fed with a black rice bran-supplemented diet [32]. Our *in vitro* study shows that a similar effect can be also obtained with extracts from other rice varieties in commercially available forms, such as integral rice, black and red rice, and a common by-product such as bran obtained from white rice varieties, suggesting it's possible as functional food.

Figure 5. Expression level of iNOS protein. Mouse RAW 264.7 macrophages pre-treated for 16 h with 125 μM of extracts obtained from integral, red, black rice and bran followed by exposure to 100 ng/mL LPS for additional 24 h. iNOS protein level was determined by Western blot using a polyclonal antibody anti-iNOS (Ab160862, Cayman chemical). As control, a monoclonal anti- Tubulin (AbT6074, Sigma) has been used.

4. Conclusions

Overall, our results showed that pigmented rice varieties

possessed high antioxidant capacity and high flavonoid and polyphenol content. Anthocyanins were mainly present in black rice, whereas alkylresorcinols were found only in red rice. Rice bran proved to be particularly rich in carotenoids. In terms of health benefits, we demonstrated that, upon inflammatory stimulus, extracts obtained from integral, black, red rice and bran specifically reduced the expression of iNOS, responsible for the NO production, without affecting cytokines secretion. These results indicate the capacity of rice extracts to modulate the inflammatory process, without abolishing the acute inflammatory response in case of pathogen infections.

Acknowledgements

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