The Bio-Functional Properties of Pigmented Cereals may Involve Synergies among Different Bioactive Species

The bio-functional properties of pigmented cereals relate to synergies among different bioactives

Parisa Abbasi Parizad[1], Jessica Capraro[1], Alessio Scarafoni[1], Francesco Bonomi[1], Massimo Blandino[2], Mauro Marengo[1], Debora Giordano[2], Aristodemo Carpen[1], Stefania Iametti[1]

[1] University of Milan, Department of Food, Environmental and Nutritional Sciences (DeFENS), via G. Celoria 2, 20133, Milan, Italy

[2] University of Turin, Department of Agricultural, Forest and Food Sciences (DISAFA), Largo Paolo Braccini 2, 10095, Grugliasco, Italy

ABSTRACT: This study was aimed at characterizing the anthocyanin profile in different varieties of pigmented corn and wheat and in some of their milling fractions, and at investigating the anti-inflammatory and enzyme-inhibiting activities of these materials. Acid/ethanol extracts were used to assess total anthocyanins, overall antioxidant activity, the overall polyphenol profile, and for evaluating the inhibition of pancreatic alpha-amylase and of intestinal alpha-glucosidase. Dose-dependent inhibition of both enzymes was evident in all extracts within the same range of bioactive concentration, although with a different efficiency of individual extracts towards each enzyme. Anti-inflammatory response was evaluated by using acid-free extracts and a cellular model based on Caco-2 cells transiently transfected with a luciferase reporter gene responding to cytokine stimulation. The immune response of interleukin-stimulated cells decreased significantly in a dose-responsive manner in the presence of 20-50 micromol/liter anthocyanins from all grains extracts, again with a different efficiency. By comparing the different inhibitory ability of extracts from the various sources, it appears that the observed effects are in most cases higher than what observed in similar extracts from other sources, and that the activity in each extract may be related to specific synergies between anthocyanins and other phenolics.

Keywords: pigmented cereals; anthocyanins; polyphenols; anti-inflammatory activity; inhibition of carbohydrate metabolism enzymes

Abbreviations: PWD1, Purple Wheat (bioactives-rich fraction from a debranning step); BWSK, Blue Wheat (bioactives-rich fraction from a debranning step); BCT, Blue Corn (variety T, whole meal); BCMF, Blue Corn (variety MF, whole meal); Rostrato, red corn (variety Rostrato, whole meal); TAC, Total Anthocyanin Content; TPC, Total Polyphenol Content; FRAP, Ferric Reducing Antioxidant Power.

INTRODUCTION

Colored cereal grains contain a high amount of phenolics and flavonoids, including anthocyanins (Abdel-Aal, Young and Rabalski, 2006; Liu, Qiu and Beta, 2010), that are responsible for the blue, purple or red color of the grains. Color and intensity of the pigmentation depend on the content and relative abundance of specific anthocyanins compounds, that may be present in various anatomical regions of the kernel. A purple color is mainly due to cyanidin and to its 3-O glucoside, typically found in the pericarp layer. Delphinidin and its derivatives - often found also in the aleuronic layer - are responsible for the blue color, whereas high concentrations of pelargonidin and of catechin-based tannins gives a red hue to wheat and corn seeds (Abdel-Aal, Young and Rabalski, 2006; Abdel-Aal, Abou-Arab et al. 2008; Hosseinian, Li et al. 2008; Ficco, Mastrangelo et al. 2014).

In vitro and *in vivo* studies have proven the antioxidant activity of these compounds and their capacity to prevent human health conditions linked to oxidative stress (Masisi, Beta and Moghadasian 2016). A number of reports also have associated anthocyanins and other phenolic compounds to anti-inflammatory activities. In fact, these bioactive compounds and their metabolites can act on endogenous cell signaling pathways, on gene expression, and on the gut microbiota, decreasing inflammatory responses through a mechanism that primarily involves modulation of various steps in the NF-kB signaling pathways (Romier, Schneider et al 2009; Vendrame and Klimis-Zacas 2015). The ability of anthocyanins to suppress inflammatory responses is closely related to their chemical structure (Gonzales, Smagghe et al 2015).

Polyphenols and anthocyanins also have been shown to inhibit the enzymatic activity of pancreatic α-amylase and of brush-border α-glucosidase. Both enzymes have a fundamental role in carbohydrates metabolism (Nunes, Ferreira et al 2013). In particular, the inhibition of intestinal α-glucosidases could postpone the digestion and absorption of carbohydrates, with a decrease of postprandial hyperglycemia. The delayed action of the inhibited enzyme is considered one of the most effective approaches to control type-2 diabetes (McDougall and Stewart, 2005, Gowd, Jia and Chen 2017).

Studies on the bioactive role of anthocyanin-rich food have been focused mainly on grapes and berries (Harsha, Gardana et al 2013; Del Bo, Martini et al 2015), as these fruits are possibly the richest sources of these compounds (Wu, Beecher et al 2006). A number of reports also have shown that various phenolics in grape skin (Lavelli, Harsha et al 2016) or in soft fruits (McDougall, Shpiro et al 2005) inhibit pancreatic α-amylase and brush-border α-glucosidase. Incorporation of grape and berry phenolics in various foods under conditions allowing retention of their biological activities has been the subject of several investigations (for a recent review, see Martins, Pinho and Ferreira, 2017).

On the contrary, investigation on pigmented cereals are somewhat limited (Angelino, Cossu et al 2017; Masisi, Beta, and Moghadasian 2016), even though the anthocyanin content in some varieties of these species may come close to that of some grapes and berries (Wu, Beecher et al 2006). In the case of

several grains, anthocyanin-rich fractions may be obtained from appropriate debranning procedures prior to milling (Giordano et al 2017). These fractions from pigmented grains - that are usually discarded in the case of non-pigmented grains - are also rich in fiber and minerals, and could represent suitable ingredients for the production of staple foods such as pasta, bread, and other baked goods. For instance, a fiber- and polyphenols-enriched pasta has been obtained by incorporating nutritionally significant amounts (25% w/w) of a cortical fraction from the debranning of pigmented grains (Zanoletti, Parizad et al 2017). Also reported are procedures for the production of other cereal-based foods from similar sources (Pasqualone, Bianco et al 2015; Ficco, De Simone et al 2016). These procedures are taking advantage of the reportedly modest sensitivity of grain bioactives to some of the most common processing steps (Li, Pickard and Beta 2007; Lopez-Martinez, Parkin and Garcia 2011).

This study was aimed at: 1) characterizing the major anthocyanins and phenolics in different types and varieties of pigmented cereals; 2) assessing the capacity of the various extracts to inhibit the activity of partially purified pancreatic alpha-amylase and brush-border alpha-glucosidase, that represent key enzymes in carbohydrate digestion and uptake; 3) defining the anti-inflammatory properties of cereal-derived phenolics- and anthocyanin-rich extracts by using appropriate cellular models. The information provided by this comparative investigation may offer some useful hints for identifying the best pigmented grains to be used for developing dietary supplements and/or functional foods.

MATERIALS AND METHODS

Grain samples

Three corn varieties and two wheat varieties of wheat (all locally sourced, unless otherwise indicated) were used in this study: blue corn T (BCT), cultivar Indigo Blue, courtesy of Clarkson Grain (Illinois, USA) ; blue corn MF (BCMF), courtesy of Molino Favero (Padova, Italy); Rostrato red corn (Rostrato, courtesy of Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro Cerealicoltura e Colture Industriali, CREA-CI, Italy); an anthocyianin-rich fraction from debranning of blue wheat (BWSK, cv. Skorpion, courtesy of the Agricultural Research Institute Kromeriz, Ltd., Czech Republic); an anthocyianin-rich fraction from debranning of purple wheat (PWR1; Zanoletti, Parizad et al 2017). All samples were milled to fine flour (< 0.5 mm) in a lab-scale mill, and stored at 4 °C until use.

Chemicals and Enzymes

Unless otherwise specified, all chemicals were from Sigma-Aldrich (Milan, Italy), including the standards used for chromatographic profiling, the substrates for enzymatic assays, and the two enzymes used in this study (namely, rat intestinal acetone powders of alpha-glucosidase [EC 3.2.1.20], and porcine pancreatic alpha-amylase [EC 3.2.1.1]).

Preparation of extracts

A two-gram aliquot of finely ground sample was treated overnight with 15 ml of petroleum ether to remove lipids, and then centrifuged at 5000 × g for 20 minutes at 10˚ C. The solids in the pellet were dried under a nitrogen flow, and then extracted overnight under continuous mild shaking at room temperature with 15 ml of an ethanol/HCl mixture (65 volumes of 95% ethanol and 35 volumes of aqueous 0.3 M HCl). The extraction procedure was repeated twice. The supernatants from centrifugation (5000 × g for 20 minutes at 10˚ C) after each extraction step were pooled, and stored at 4°C. These extracts were used without further treatments for analytical measurements and for HPLC profiling. Extracts to be used in experiments with Caco-2 cells were prepared through a similar procedure, in which water was substituted for 0.3 M HCl in the extraction mixture to avoid interference with the cellular assays as well as cell viability issues. When required, solvent was removed from these extracts under vacuum. Also, please note that the substitution of ethanol for the methanol most commonly used in the preparation of this type of extracts relates to having observed a toxic effect of residual methanol on Caco-2 cells in preliminary experiments.

Total anthocyanins

The total anthocyanins content (TAC) in individual ethanol/HCl extracts was determined by using a pH differential method (Hosseinian, Li, and Beta 2008). The original extracts were diluted in either 30 mM KCl/HCl buffer, pH 1.0, or in 400 mM sodium acetate, pH 4.5. Then, the absorbance of each sample at the two pH values was measured at 520 nm against water, and the readings corrected for haze (measured as absorbance at 700 nm). Results are expressed as micrograms of cyanidin 3-O-glucoside equivalents per gram of dry sample flour.

Total phenolics

The total phenolics content (TPC) in individual ethanol/HCl extracts was measured using a Folin-Ciocalteu method (Singleton, Orthofer, and Lamuela-Raventós 1999). An aliquot of each ethanol/HCl extract (0.1 mL) was mixed with 0.500 mL of Folin-Ciocalteu reagent and 2 mL of 15% (w/v) sodium carbonate, and brought to a final volume of 10 mL by adding distilled water. The mixture was allowed to rest in darkness at room temperature for 1 hour, and then was centrifuged at 12000 rpm for 10 min. The absorbance of the supernatant was read at 765 nm against water. The results were expressed as mg gallic acid equivalents per gram of dry sample.

Ferric reducing-antioxidant power (FRAP)

The FRAP assay was performed according to Benzie and Strain (1996). The FRAP reagent was prepared by mixing 25 mL of 300 mM acetate buffer, pH 3.6, 2.5 mL of 10 mM 2,4,6-tripyridyl-*s*-triazine in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3$. An aliquot of each extract was diluted as appropriate with the same

ethanol/HCl mixture used for the preparation of extracts. The diluted extract (0.4 mL, five dilutions in duplicate) was mixed with 3 mL of the FRAP reagent. Samples were incubated at 37 °C for 4 minutes, and their absorbance was measured at 593 nm against a blank containing 3 mL of the FRAP reagent and 0.4 ml of the ethanol/HCl mixture. A calibration curve was constructed by using a solution of $FeSO_4 \cdot 7H_2O$ in methanol. Results are expressed as microequivalents of Fe(II) sulfate per gram of dry sample.

Anthocyanin and phenolics profiling by RP-HPLC

RP-HPLC profiling was performed on a Waters 600 E HPLC, equipped with a Waters 717 auto-sampler and a Waters 996 PDA detector (Waters, Milan, IT), by using a C18 column (5 µm, 4.6 × 250 mm, Waters, Milan, Italy). Typically, 0.1-0.2 mL of individual ethanol/HCl extracts were loaded on the column. Elution was carried out at 0.8 mL/min, using a linear gradient from 100% A (0.1% trifluoroacetic acid in water) to 100% B (0.1% trifluoracetic acid in acetonitrile), with the following gradient program: 0 to 5 % B in 5 min; 5 to 40 % B from 5 to 40 min; 40 to 70% B from 40 to 48 min, followed by a wash with 100% B. The absorbance of the eluate was monitored at 520 nm (for anthocyanins), at 350 nm (for rutin and quercetin), at 320 nm (for ferulic acid), and at 280 nm (for catechin and epicatechin). Identification of the separated anthocyanins was achieved by combining chromatographic data and UV–Vis spectra. Quantification was based on calibration curves obtained from individual solutions of each of the following standards: cyanidin chloride, delphinidin chloride, rutin, quercetin, catechin, epicatechin and ferulic acid. For each compound, results are expressed as mg/kg of original sample.

Immunomodulatory properties of extracts

Cultivation of Caco-2 cells. Human intestinal epithelial Caco-2 cells were kindly provided by Prof. Maria Rosa Lovati (Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano). Cells were grown in 75 cm^2 flasks at 37 °C in a humidified atmosphere (95% air and 5% CO_2) using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The day before transfection, cells were seeded in 24-well plates at a density of 2×10^5 cells/cm^2.

Transient Transfection. Caco-2 cells were transiently transfected with the pNiFty2-Luc plasmid (InvivoGen, Rho, Italy). This plasmid combines five NF-κB binding sites along with the luciferase reporter gene *luc*, in an arrangement that stimulates the expression of the luciferase gene in the presence of NF-κB activating molecules. Transfection was performed by using the StoS transfection kit (GeneSpin, Milan, Italy), following the supplier's protocol. After transfection, cells were grown in complete DMEM medium as described before.

Immunomodulation assay. The immunomodulation assay was performed 24 hours after transfection. Transfected Caco-2 cells were incubated in 0.2 ml of DMEM containing various concentrations of

anthocyanins (0.025, 0.050 and 0.075 mM, expressed as cyanidin 3-O-glucoside) from each individual extract, and Interleukin 1β (IL) at a final concentration of 20 ng/mL. After 4 hours at 37° C, the plate was chilled on ice for 15 minutes. Cells were scraped from the bottom of the wells, and the content of each well was transferred into 1.5 ml Eppendorf tubes. After sonication for 10 seconds using a Soniprep 150 Ultrasonic Disintegrator (MSE, Fisher Scientific, Loughborough, UK), insoluble materials were removed by centrifugation. A 0.1 mL aliquot of each supernatant was placed in a microtiter plate well, to which ATP (0.012 mL, 10 mM) and D-luciferin (0.012 mL, 0.1 mM) were added. The emitted bioluminescence was monitored every 120 s using a VICTOR3 1420 Multilabel Counter (PerkinElmer, Waltham, MA), and compared to that of a reference sample containing no anthocyanins.

Cell Viability. Following treatment with individual ethanolic extracts under the conditions outlined above, cells were detached from the well bottom by treatment with a trypsin-EDTA solution for 5-8 minutes. The suspension was centrifuged at low speed, and the cells re-suspended in an appropriate volume of complete medium. Aliquots of the suspension were mixed with an equal volume of 0.4 % (w/v) Tryphan Blue, and cell viability assessed by using a TC20 cell counter (BioRad, Segrate, Italy).

Alpha-glucosidase inhibition

Rat intestinal acetone powder (200 mg) was suspended in 4 mL of ice-cold phosphate buffer (50 mM, pH 6.8) and sonicated for at least 15 min at 4 °C. The suspension was then vortexed and centrifuged (10,000 × g, 4 °C, 30 min) to obtain the supernatant used for subsequent assays. Assays were set up by mixing 0.65 mL of 50 mM phosphate buffer, pH 6.8 with 0.1 mL of the enzyme solution and with 0-0.05 mL of individual ethanol/HCl extracts. After pre-incubation for 5 min at 37 °C, the reaction was started by adding 0.2 mL of 1 mM p-nitrophenyl glucoside and allowed to continue for 25 min at 37 °C. The mixture was then centrifuged (10,000 × g, 10 min) and the absorbance of the supernatant was measured at 405 nm. Blanks were prepared in the absence of enzyme and of the ethanol/HCl mixture, and controls were otherwise complete reaction mixtures, containing appropriate volumes of ethanol/HCl, but no bioactives. Tests were performed in triplicate for each dilution of the original extract. Results were compared with those obtained by using known amounts of acarbose (from a stock solution in ethanol/HCl) as the reference inhibitor.

Alpha-amylase inhibition

A 0.2 mL aliquot of an enzyme stock solution (0.01 mM in 50 mM phosphate buffer, pH 6.8) was diluted with 0.55 mL of the same buffer, and mixed with 0.05 mL of individual ethanol/HCl extracts, diluted as appropriate in ethanol/HCl. After pre-incubation for 5 min at 37 °C, the reaction was started by adding 0.2 mL of 1 mM p-nitrophenyl glucoside and allowed to continue for 25 min at 37 °C. The mixture was then centrifuged (10,000 × g, 10 min), and the absorbance of the supernatant was measured at 405 nm. Blanks were prepared in the absence of enzyme and of the ethanol/HCl mixture. Controls were otherwise

complete reaction mixtures, containing appropriate volumes of ethanol/HCl, but no bioactives. Tests were performed in triplicate for each dilution of the original extract. Results were compared with those obtained by using known amounts of acarbose (from a stock solution in ethanol/HCl) as the reference inhibitor.

Statistical analysis

All data were analysed by one-way ANOVA, using the least significant difference ($p < 0.05$) as a multiple range test. Results are reported as the average ± SD. The built-in non-linear regression routines in SigmaPlot (rev 10, Jandel Scientific, San Rafael, CA) were used for graphical analysis of the results.

RESULTS AND DISCUSSION

Total anthocyanins and total polyphenol content, and total antioxidant activity

As shown in Table 1, the Total Anthocyanin Content (TAC) varied sensibly among the various grains, confirming previous reports (Abdel-Aal, Young and Rabalski 2006). The fraction derived from the debranning of purple wheat (PWR1) had the highest TAC, closely followed by whole meals from BCT and BCMF. The fraction obtained from debranning of Blue Wheat SK (BWSK) and the Rostrato corn whole meal had the lowest TAC values.

A comparison with literature data (Wu, Beecher et al 2006) indicates that even the highest TAC values found in the materials under scrutiny here are far below those reported for some grapes (1.2 mg/g whole fruit in Concord grapes) or in cultivated blueberries (about 4 mg/g whole fruit), but it is of interest that the richest fractions - at least in the case of purple wheat - correspond to materials that are typically considered byproducts when wheat is processed into refined flour, and therefore are not used for direct human consumption. A study on anthocyanin profile in various grain tissues (Abdel-Aal, Young and Rabalski 2006) indicated that TAC in purple wheat bran was in the 0.45 mg/g range, roughly twice what measured by the same Authors in blue wheat bran. In those studies, TAC in the combined whole bran and milling shorts from purple wheat was in the 0.17 mg/g range, whereas TAC blue wheat middlings was in the 0.03 mg/g range. TAC in the aleuronic layer from blue wheat did not exceed 0.25 mg/g (Jaafar, Baron et al 2013). By comparing these values with those in the specific debranning fractions used in this study, the advantage of introducing an appropriately designed debranning step as for obtaining anthocyanin-rich materials from grains that contain anthocyanins only in their outer layer may be seen as self-evident.

The TAC of the blue corn varieties investigated here (both BCT and BCMF) was almost twice that reported in previous studies on similar grains (Nankar, Dungan et al 2016). Whether this relates to the use of different solvents for anthocyanidin extraction in these studies, to the remarkable cultivar-related

variability (Nankar, Dungan et al 2016), or to the effects of breeding conditions (Harakotr, Suriharn et al, 2014) remains to be ascertained.

The Rostrato corn had the lowest TAC among all the grains characterized in this study. However, the total polyphenol content and the overall antioxidant activity in extracts from the whole flour of Rostrato corn were much higher than those found in equivalent extracts from other pigmented corn varieties. TPC and FRAP values for Rostrato red corn were indeed comparable to those measured in extracts from purple wheat debranning fractions. Analysis of the data in Table 1 shows a positive correlation ($R^2 = 0.970$; $P < 0.001$) between TPC and antioxidant activity (expressed as FRAP) in all samples see Fig. 1S). However, the antioxidant activity of individual phenolic compounds in biological systems is reportedly dependent on their chemical structure (Marko, Puppel, et al 2004), and a detailed chemical profiling of the various extracts is required to assess the chemical basis for their different antioxidant capacity.

[Table 1 here]

Identification of anthocyanins and phenolics by HPLC

Anthocyanins and phenolic compounds in the various extracts were separated by HPLC, and were identified and quantified through comparison with appropriate standards. Results of these profiling attempts are shown in Table 2, that presents a quantitative analysis of those compounds that could be identified unequivocally. Indeed, caution should be used when interpreting these data, as even very extensive phenolics profiling carried out by most updated methodologies (Siebenhandl, Grausgruber et al 2007; Ficco, Mastrangelo et al 2014) did not allow straightforward structural identification of 40-70% of the total anthocyanins present in grains and grain-derived foods.

[Table 2 here]

With these limitations, the data in table 2 point out to some obvious and some unexpected differences in the anthocyanin profile. Despite the use of different extraction protocols and solvents, the values presented in Table 2 of the current study are reasonably close to those reported for similar - although not identical – grains (Abdel-Aal, Young and Rabalski 2006; Giordano et al. 2017). Taking into account only blue colored grains, the cyanidin/delphinidin ratio (considering both the glycosylated and non-glycosylated forms of cyanidin) ranged from 0.5 (BCMF) to 1.7 (BCT) in corn flours, and from 1.3 (PWD1) to 5.4 (BWSK) in wheat samples. This may be of some relevance, in consideration of the structural and reactivity differences among the two classes of anthocyanins.

As for other phenolics identified in our HPLC profiling, we found anthocyanin-poor extracts from BCT to be the richest in ferulic acid, whereas extracts from BWSK and Rostrato contained the highest amounts of rutin and catechin. PWD1 extracts had by far the highest content in quercetin and the lowest in

rutin, as well as high epicatechin levels. However, epicatechin levels in extracts from the red Rostrato corn wholemeal were 4-fold higher than in PWD1 extracts (and 10 to 15-fold higher than those in extracts from wholemeal from both the blue corn varieties considered here).

As stated above, these findings do not provide a comprehensive analytical profiling of each and every compound in these extracts. Rather, these data confirm that the absolute amounts of individual components (and their ratios) are greatly different among the various grains and their varieties within a given species. Such variability should be taken into account when trying to correlate compositional data of individual extracts with properties measured by assessing their functionality by using either chemical or enzymatic assays or on highly sensitive cellular models.

Inhibition of intestinal α-glucosidase and pancreatic α-amylase activities

As pointed out in many studies (for a recent review see Gowd, Jia and Chen 2017), the ability of anthocyanins to inhibit the activity of enzymes involved in glucose breakdown and uptake may represent a useful strategy in managing post-prandial levels of blood glucose. In this study, the inhibitory activity towards alpha-glucosidase and alpha-amylase was evaluated in vitro by using the same ethanol/HCl extracts characterized in Table 1. Acarbose - a synthetic inhibitor of these activities, that is commonly used to manage type-2 diabetes - was used as the reference inhibitor (Lavelli, Harsha et al 2016).

As shown in the two panels of Fig. 1, all extracts gave dose-dependent inhibition of both enzymes. Please note that the actual amount of extracts used in these assays was variable, as the concentration of bioactive species in assays was normalized for the total anthocyanin content of individual extracts. All extracts, with the only exception of the one from BWSK, inhibited brush-border alpha-glucosidase more effectively than equivalent concentrations of acarbose, and extracts from the Rostrato red corn whole meal proved to be - by far - the most efficient. Acarbose was the most efficient inhibitor of pancreatic alpha-amylase, at least at concentrations below 100 mg/L. In the case of alpha-amylase, the inhibitory efficacy of extracts was found to increase in the order: BWSK≤BCT<Rostrato≤BCMF<PWD1.

[Figure 1 here]

For both enzymes, we tried to correlate the inhibition efficiency with the profile of bioactives in each ethanolic/HCl extract used for enzymatic assays, as inferred from the information reported in Table 2. However, these attempts were inconclusive, if not for cyanidin (see Fig. 2S). Also, no straightforward relationship was evident among TAC, TPC, or FRAP in individual extracts and the observed inhibition efficacy. This is consistent with previous observations on the ability of anthocyanidins (either as aglycones or in various glycosylated forms) and of various types of phenolics in inhibiting both these enzymes as well as other proteins involved in glucose uptake (Malunga, Eck and Beta 2016; Gowd, Jia and Chen 2017). Similar results were obtained in experiments with isolated compounds, aimed at assessing possible

structure/function relationships in this class of compounds (McDougall, Shpiro et al 2005; Gonzales, Smagghe et al 2015).

A comparison of our data with literature reports also indicates that the inhibitory effects observed with the cereal extracts used here (on a comparable anthocyanidin content) is sensibly higher than that reported on similar extracts from a variety of pigmented materials from different plant sources. On an anthocyanin content basis, the values of I_{50} measured in this study are in the 40-100 mg/L range, that is, about half those reported in previous studies with purified anthocyanins (see, for instance, Akkarachiyasit, Charoenlertkul et al 2010). This may be taken as an indication of synergistic effects between anthocyanidins and phenolics in the extracts used in this study, as already hypothesized for phenolics and anthocyanins from other sources (McDougall and Stewart 2005; Boath, Stewart and McDougall 2012). However, getting some molecular clues on the nature of the compounds involved in this hypothetical synergy and on the specific concentration ranges required for optimum activity will only be possible - in the case of pigmented grains - when complete compositional profiles will become available.

Anti-inflammatory properties of pigmented grains extracts

Anti-inflammatory effects of anthocyanins and polyphenols from different fruits and grains have been studied *in vitro* and *in vivo* (Vitaglione, Mennella et al 2015). Both classes of compounds were found able to decrease inflammation by modulating various steps of the NF-κB signaling pathways (Romier, Schneider et al 2009; Taverniti, Fracassetti et al 2014; Lee, Bohkyung et al 2014; Vendrame and Klimis-Zacas 2015).

In this study, the anti-inflammatory properties of anthocyanins-rich extracts of the pigmented grains were compared by determining the expression of NF-κB in Caco-2 cells upon stimulation with interleukin 1β. In these assays, we used extracts prepared without the addition of HCl to aqueous ethanol, for the reasons reported under Materials and Methods. Compositional differences in extracts as related to the presence/absence of HCl are reported in Table 1S. In each extract, the actual concentration of total anthocyanins was expressed as cyanidin 3-O-glucoside equivalents (CGE), in order to normalize the different anthocyanin profiles of individual extracts. None of the extracts was found to affect cell viability under the conditions used in this study.

As shown in Fig. 2, anthocyanin-rich ethanol-only extracts from all grains were effective to decrease the inflammatory response in a dose-dependent manner. At the highest concentration used in this study (75 μM as CGE, see above), extracts from pigmented wheat gave almost complete disappearance of NF-κB activation by IL 1-β (99.9% and 99.8%, respectively, for PWD1 and BWSK), but significant inhibition was observed also for extracts from the corn varieties Rostrato (94.5%) and BCMF (92.1%), whereas BCT extracts were the least effective (66.2%).

[Figure 2 here]

From the data in Fig 2, 50% inhibition of NF-κB activation by IL 1-β required CGE levels in the assay around 20 μM (for PWD1, BWSK, and Rostrato), whereas much higher concentrations were required for BCMF (around 50 μM) and BCT (around 65 μM). The concentrations reported in Fig. 2 for decrease in inflammatory response are lower than literature data on anthocyanin-rich fractions from blueberries, that were found effective - under very similar assay conditions - at concentrations of anthocyanins in the 50-100 microgram/ml range, corresponding to roughly 100-200 micromolar CGE (Taverniti, Fracassetti et al, 2014).

Since the results presented in Figure 2 were obtained under normalized anthocyanin concentration, we tried to assess whether the composition of these extracts was related to the inhibition of inflammatory response. It must be remembered that all the inhibition assays were carried out with volumes of extracts containing identical amounts of anthocyanins, so that the different response evident from Fig. 1 should be related to the different composition of individual extracts (see Table 1S), either in terms of their different anthocyanin profile or in terms of their different contents (and different relative concentrations) of the various bioactives.

Due to the inherent difficulties of profiling individual anthocyanins and phenolics in extracts from pigmented grains, as discussed above and as reported in the literature (Siebenhandl, Grausgruber et al 2007; Ficco, Mastrangelo et a, 2014), we took a conservative approach in the analysis of these data, and considered only the overall compositional data (i.e., TAC, TPC, and FRAP). As made evident by the data presented in Fig. 3, a decrease in I_{50} of the inflammatory response corresponded to increasing TPC/TAC ratios in the various extracts.

[Figure 3 here]

The evidence presented in Figure 3 may be taken as an indication of a possible synergistic effect between anthocyanins and phenolic components. Whether this relates to a protective effect of phenolics against intracellular oxidative degradation of anthocyanins (Nunes, Ferreira et al, 2013) or to other - more specific - interactions which will be the subject of future studies. In this frame, it is noteworthy that a decrease in I_{50} was also evident at increasing FRAP/TAC ratios in individual extracts (see Fig. 3S). However, this relationship is not surprising, given that the FRAP capacity of the extracts used in the experiments involving Caco-2 cells was - expectedly - correlated to their TPC (see Fig. 1S and Table 1S), as already observed for the extracts prepared in the presence of HCl, and discussed above when commenting Table 1.

CONCLUSIONS

Our study confirms that anthocyanins and phenolic compounds in pigmented cereals are present in concentrations high enough for considering these raw materials as good sources of bioactives. Although to a different extent, all the grains extracts characterized in this study showed anti-inflammatory and enzyme inhibiting properties. Thus, these grains appear suitable as ingredients for the direct transformation into

foods like bread, noodles, or pasta, or as possible additives in dairy products or beverages, in particular when association with high levels of other bioactives (such as dietary fiber) is of relevance. In this frame, it is of interest that both anti-inflammatory and enzyme inhibiting activities were high in materials (such as the fractions obtained from debranning of pigmented wheat) that are rich in fiber and are not commonly used for human consumption.

However, the molecular reasons for the different efficacy of the various extracts characterized in this study remain somehow elusive, as remains the reasons for several of the extracts studied here being more active (at equivalent concentration of bioactives) than extracts from other sources. Indeed, the data presented here suggest that neither the enzymatic inhibition nor the anti-inflammatory effects could be attributed to a specific component in the extracts. On the contrary, our data highlight that both effects may be ascribed to some type of synergy among components in a given extract.

Current work is aimed at using the same approaches reported here for identifying the nature of the synergistic species in these materials. Hopefully, when integrated with a thorough characterization of the phenolics profile in these material, of their physicochemical status, and of their susceptibility to transformation by the intestinal microflora, these studies will provide new - and hopefully useful - insights on the biochemical mechanisms that form the molecular basis of properties of these materials that may be relevant to human health.

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Table 1. Bioactives in pigmented grains

Source	*TAC* (mg Cyn-3-O-Glc eq/kg)	*TPC* (mg gallic acid eq/kg)	*FRAP* (mmol Fe(II) eq/kg)
PWD1	690 ± 20[a]	73.60 ± 0.44[a]	47.51 ± 10.26[a]
BWSK	170 ± 10[c]	33.04 ± 0.24[c]	17.90 ± 1.68[b]
BCT	660 ± 60[a]	30.84 ± 0.65[c]	22.08 ± 2.82[b]
BCMF	530 ± 10[b]	33.70 ± 0.50[c]	18.91 ± 1.83[b]
Rostrato	140 ± 10[c]	63.70 ± 0.79[b]	47.20 ± 3.28[a]

Data are mean values ± standard deviation. Different superscripts in a given column indicate statistically different values (p=0.01).

Table 2

Individual anthocyanins and phenolics in pigmented grains

Material	***identified anthocyanins, mg/kg***			***other identified phenolics, mg/kg of sample***				
	Cyanidin	*Delphinidin*	*Cyanidin-3-O-glucoside*	*Ferulic acid*	*Rutin*	*Quercetin*	*Catechin*	*Epicatechin*
PWD1	36.2 ± 4.1^{c}	52.4 ± 7.1^{c}	32.1 ± 2.1^{a}	18.4 ± 1.8^{d}	2.0 ± 0.5^{d}	18.7 ± 1.6^{a}	76.2 ± 5.3^{d}	121.1 ± 8.7^{b}
BWSK	19.5 ± 2.2^{d}	90.5 ± 8.2^{b}	19.4 ± 1.4^{b}	71.8 ± 3.1^{b}	63.0 ± 2.0^{a}	1.9 ± 0.3^{c}	385.6 ± 5.5^{a}	63.8 ± 3.4^{c}
BCT	201.1 ± 9.0^{a}	97.0 ± 2.8^{b}	23.2 ± 0.9^{b}	113.9 ± 3.4^{a}	10.7 ± 1.5^{c}	0.6 ± 0.1^{d}	112.8 ±2.9^{c}	31.7 ± 3.1^{e}
BCMF	27.2 ± 1.7^{b}	114.4 ± 6.4^{a}	35.2 ± 1.9^{a}	73.0 ± 2.8^{b}	26.2 ± 2.8^{b}	0.8 ± 0.3^{d}	252.5 ± 32.6^{b}	48.8 ± 3.1^{d}
Rostrato	16.1 ± 1.1^{d}	5.2 ± 0.5^{d}	3.1 ± 0.5^{c}	28.5 ± 1.5^{c}	62.8 ± 2.6^{a}	3.8 ± 0.7^{b}	284.2 ± 4.2^{b}	465.1 ± 7.9^{a}

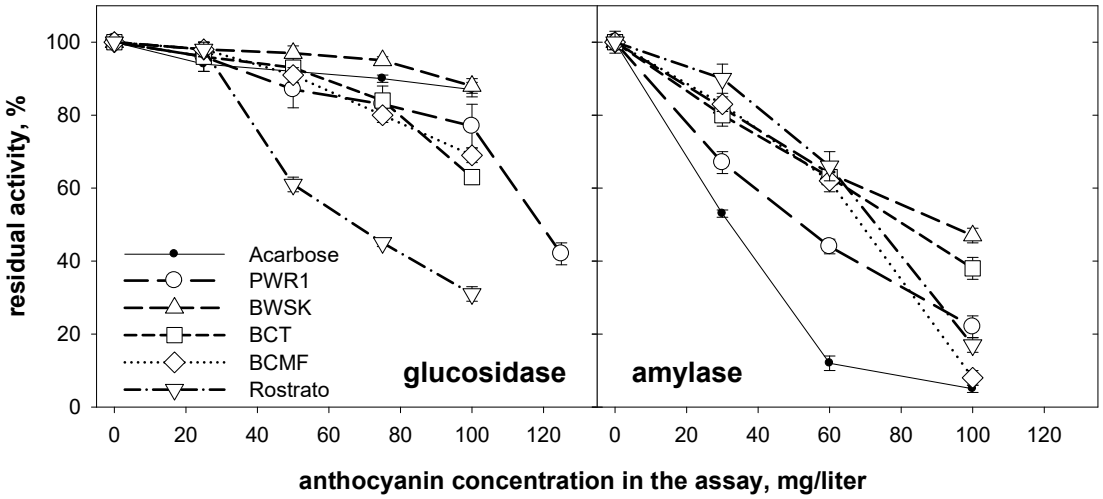
Data are mean values ± standard deviation. Different superscripts in a given column indicate statistically different values (p=0.01).

Figure legends

Figure 1. Inhibition of enzymes involved in glucose metabolism and uptake by extracts from different pigmented grains. Left panel, brush-border alpha glucosidase; right panel, pancreatic alpha amylase. Extracts are identified by the same codes used in Tables 1 and 2. Bars on each symbol indicate standard deviation (n= 3).

Figure 2. Immunosuppressive effects of the various extracts. Data are presented as percent inhibition of IL-1β-stimulated expression of NF-κB, as measured through production of a luciferase reporter. Acid-free extracts were used in these assays, and are identified by the same letter codes used in Tables 1 and 2. Bars on each symbol indicate standard deviation (n=3).

Figure 3. Dependence of I_{50} for the inhibition of NF-κB expression activation by IL 1-β on the total polyphenols content in individual extracts. For each extract, the total polyphenol content (TPC) was normalized for the total anthocyanins content (TAC). The dashed line is a best fit to an inverse second order polynomial.



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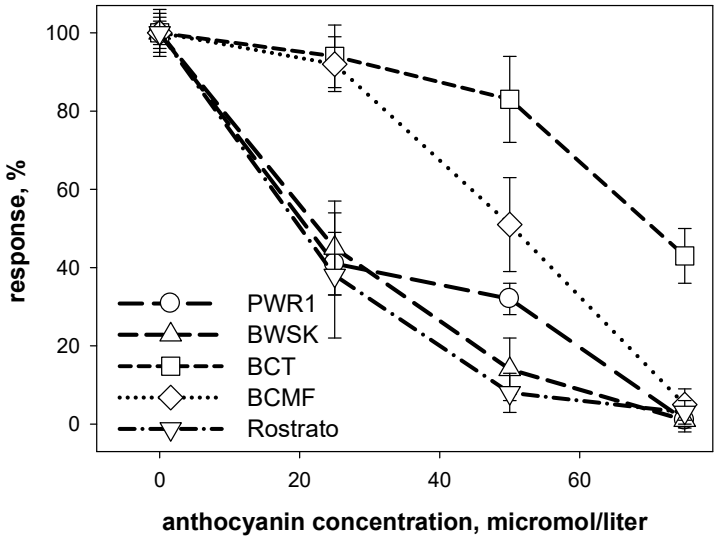

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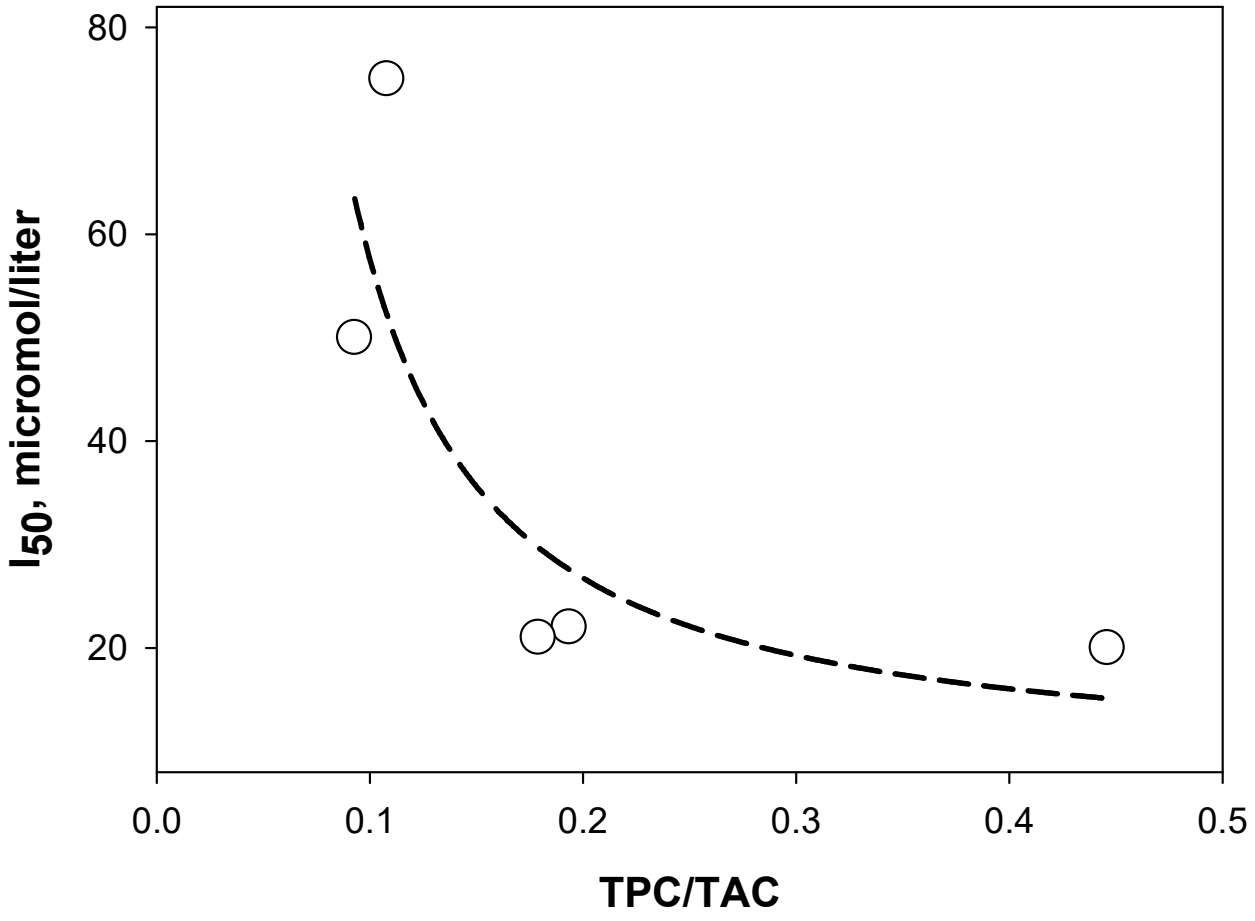

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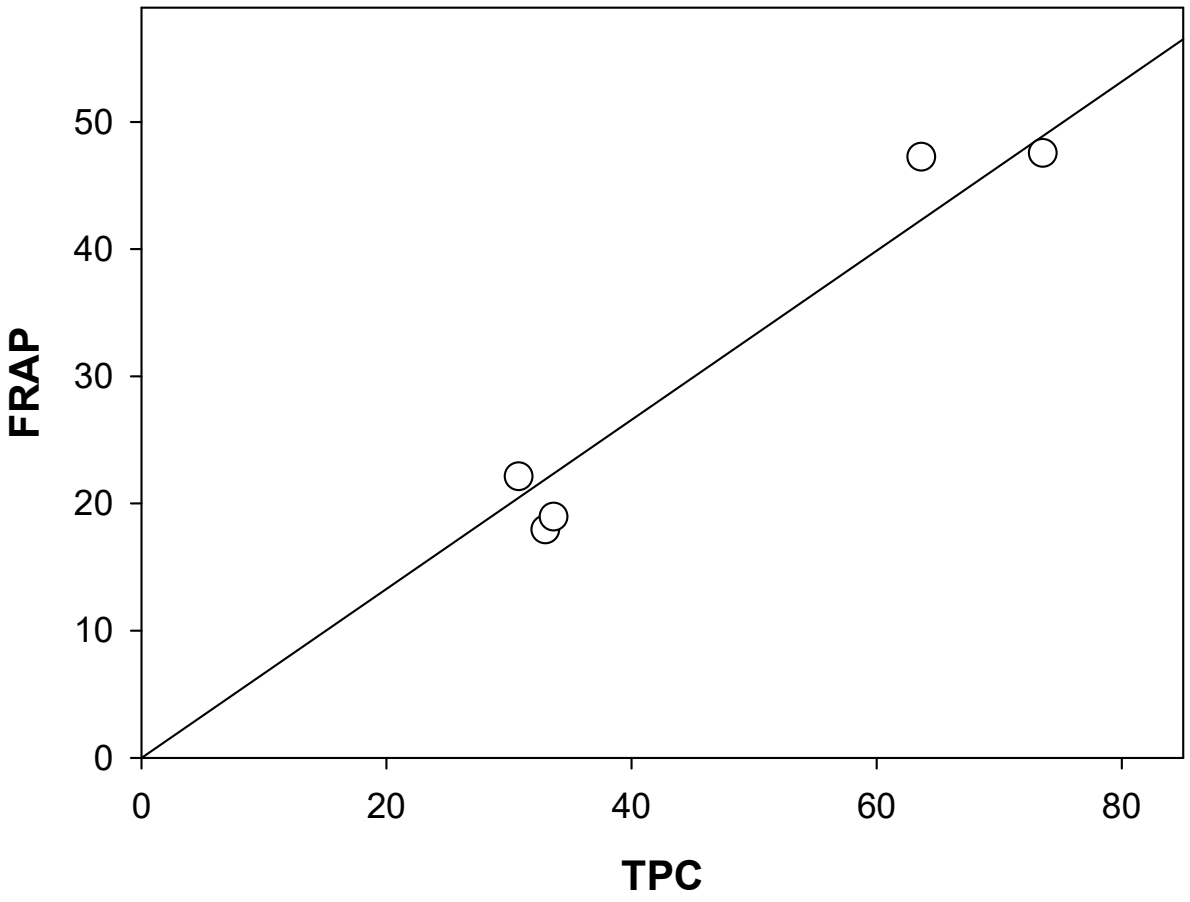

Figure 1S. Dependence of Ferric Reducing Antioxidant Power (FRAP) on the Total Polyphenols Content (TPC) of aqueous ethanol/HCl extracts.

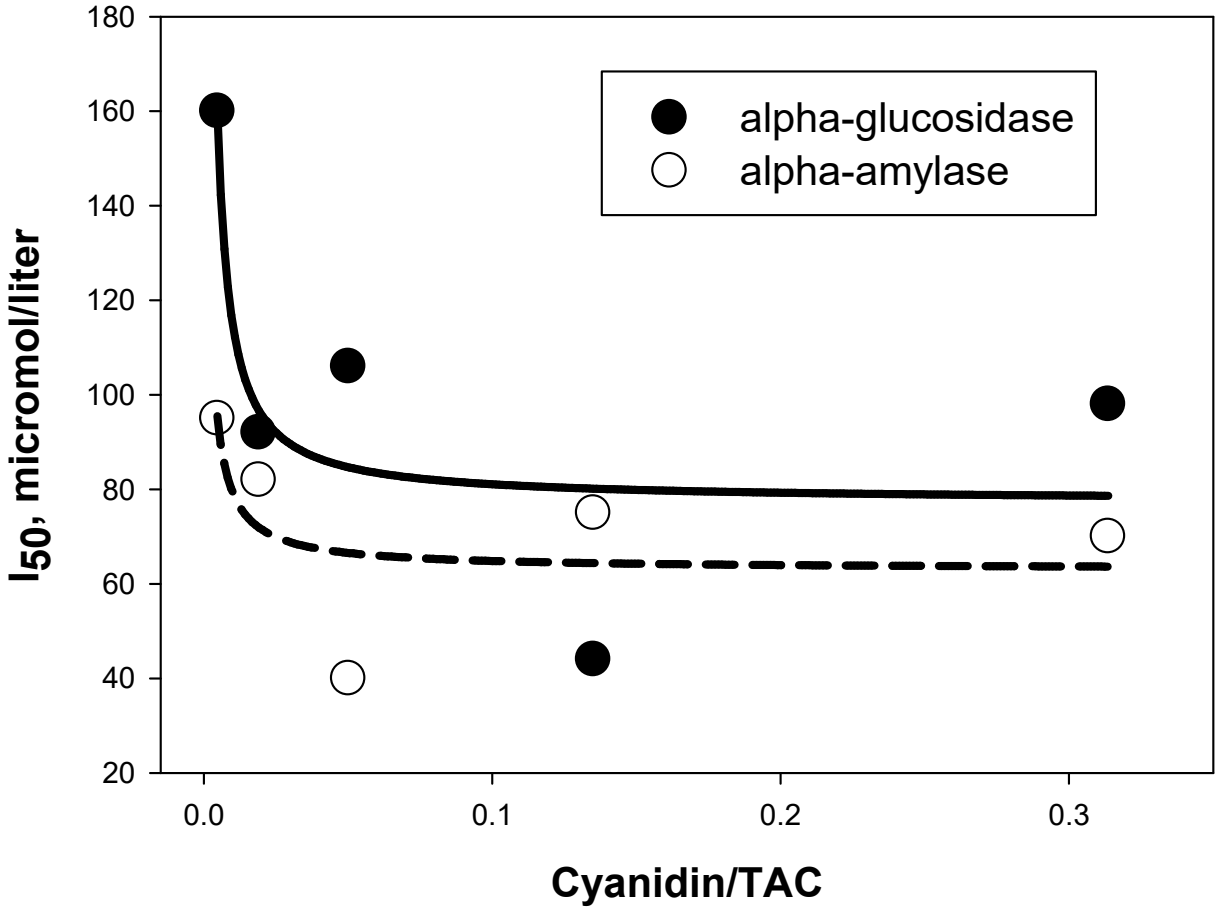

Figure 2S. Dependence of I_{50} for alpha-glucosidase and alpha-amylase on the cyanidin content in individual extracts. The cyanidin content was normalized for the total anthocyanins content in individual extracts. Lines are best fit to a three-parameters exponential decrease. Full lines, alpha-glucosidase; dashed lines, alpha-amylase.

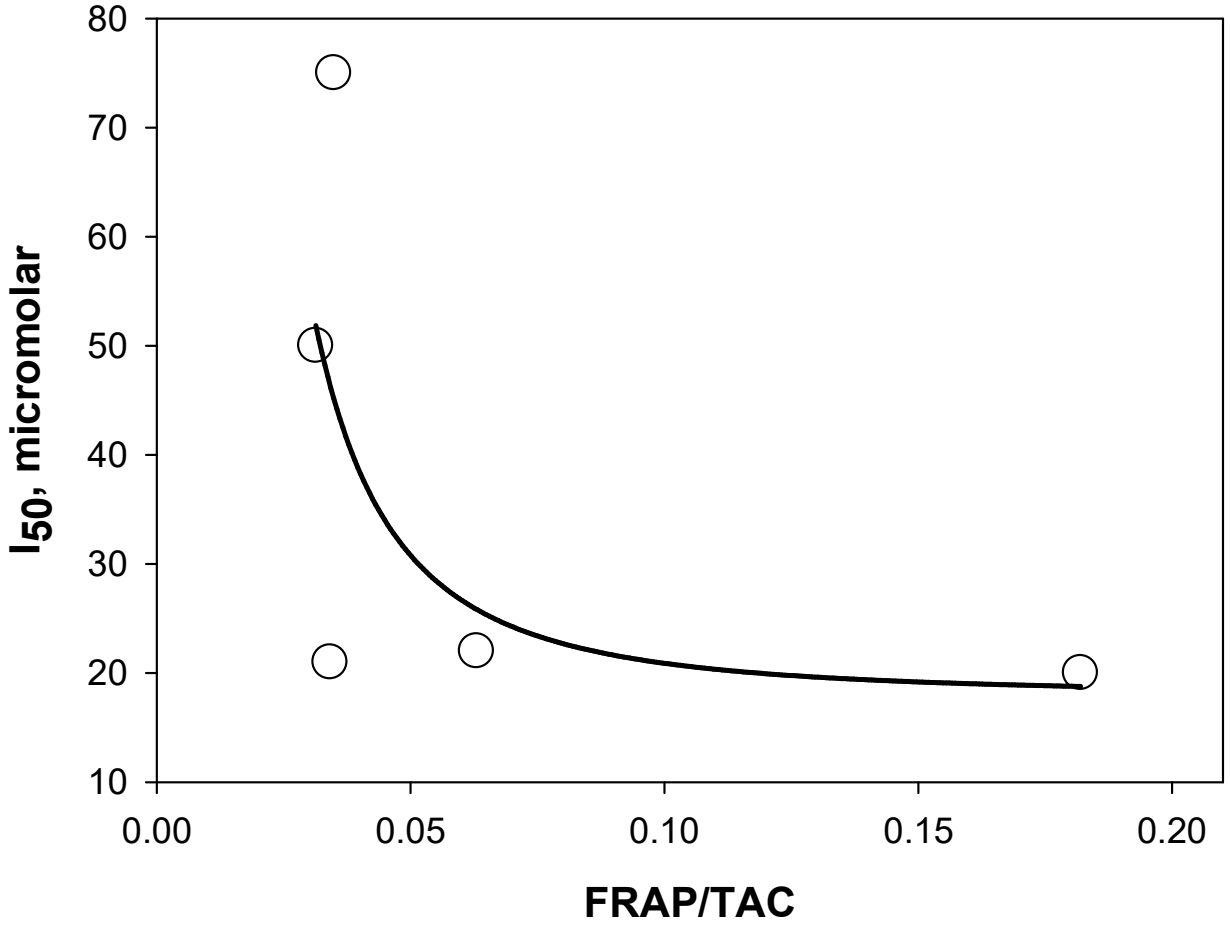

Figure 3S. Dependence of I_{50} for the inhibition of NF-κB activation by IL 1-β on the FRAP values in individual acid-free extracts. For each extract, the measured FRAP was normalized for the total anthocyanin content. The line is a best fit to an inverse second order polynomial.