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Antioxidant properties of mashua (*Tropaeolum tuberosum*) phenolic extracts against oxidative damage using biological *in vitro* assays

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ABSTRACT

Purified mashua extracts (PME) from four different coloured mashua genotypes were assayed for oxidative damage prevention. Three *in vitro* assays for oxidative damage to biological structures rich in polyunsaturated fatty acids (PUFA), such as LDL and erythrocytes, were tested: AAPH-induced TBARS assay and Cu^{2+}-induced conjugated dienes assay for LDL oxidation and AAPH-induced oxidative hemolysis of erythrocytes. Additionally, ORAC antioxidant capacity, total phenolics (TP), total flavanoids (TFA) and total anthocyanins (TA) were evaluated. In the presence of 5 μM of gallic acid equivalents (GAE), inhibitions of LDL oxidation for the PME ranged from 29.1% to 34.8% and from 51.8% to 58.1% when the TBARS and conjugated dienes assays were performed, respectively. PME inhibited the hemolysis of erythrocytes within the range 20.8–25.1%. Thus, mashua phenolic extracts are capable of scavenging peroxyl radicals, as well as chelating redox metal ions *in vitro*. ORAC and LDL protection (TBARS and conjugated dienes assays) showed good correlations with the TP and TFA, suggesting that these compounds have a good ability to protect LDL molecules under the employed conditions. In contrast, inhibition of hemolysis did not show any correlation with the evaluated phenolic assays (TP, TA, TFA) or with any of the evaluated oxidative LDL assays, suggesting a specific action of some non-evaluated compounds present in the PME. The results of this study indicate that the mashua polyphenol extracts displayed good antioxidant properties against oxidative damage in biological structures rich in PUFA. The displayed antioxidant properties could be applied in the field of food or cosmetic industry.

1. Introduction

Phenolic compounds are bioactive substances widely distributed in plants and are important constituents of the human diet. Plant phenolics comprise a great diversity of compounds, such as flavonoids (anthocyanins, flavanols, flavonols, flavones, among others) and several classes of non-flavonoids (phenolic acids, lignins, stilbenes) (Harborne, 1989).

Several works have shown that phenolic compounds play a role as antioxidants through different mechanisms of action, such as: scavenging of free radicals (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002; Zhu, Hackman, Ensunsa, Holt, & Keen, 2002), quenching of reactive oxygen species, inhibiting of oxidative enzymes (Edenharder & Grunhage, 2003; Lee, Koo, & Min, 2004; Sarni-Manchado & Cheynier, 2006), chelating of transition metals or through interaction with biomembranes (Liao & Yin, 2000). These properties make those types of compounds good candidates as potential protectors against food oxidation and biological aging of tissues (Sarni-Manchado & Cheynier, 2006). Thus, phenolic compounds could be considered as natural antioxidants with potential applications in foods or cosmetics.

Lipid oxidation is one of the major causes of chemical spoilage, resulting in rancidity and/or deterioration of the nutritional quality, colour, flavour, texture and safety of foods (Antolovich et al., 2002). The lipid oxidation process is involved in oxidative damage occurring at a cellular level that leads to aging (Lee et al., 2004). Thus, several *in vitro* antioxidant capacity assays that evaluate lipid oxidation have been designed (Antolovich et al., 2002; MacDonald-Wicks, Wood, & Garg, 2006; Sánchez-Moreno & Larrauri, 1998). In these assays a lipid or lipoprotein substrate is used under standard conditions and the degree of the inhibition of oxidation given by an antioxidant (e.g., phenolic compounds) is measured (Sánchez-Moreno & Larrauri, 1998). Besides, erythrocytes have been proposed as model substrates to evaluate *in vitro* oxidative damage

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of biological membranes (Dai, Miao, Zhou, Yang, & Liu, 2006; Lee, Krueger, Reed, & Richards, 2006).

Low-density lipoprotein (LDL) and erythrocytes, as well as many food matrices contain important amounts of polyunsaturated fatty acids (PUFA), which are vital for the normal function of biological structures, providing particular physicochemical properties. However, due to the presence of PUFA, the risk of lipid oxidation in these types of systems increases. Free radicals, such as the hydroxyl radical ($OH^{\cdot}$), the altoxyl radical ($RO^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$), initiate lipid peroxidation in biological structures rich in PUFA, leading to modifications of their normal functions/characteristics or to the formation of numerous oxidized products (MacDonald-Wicks et al., 2006; Wood, Gibson, & Garg, 2006). In addition, the formed lipid peroxides can further decompose due to reactions with metal ions (such as iron and copper) or iron proteins (such as hemoglobin) (Wood et al., 2006). Thus, the measurement of formed products from oxidized PUFA can be taken as an index of lipid oxidation.

In vitro antioxidant capacity assays based on oxidative hemolysis of erythrocytes and LDL oxidation are considered as good experimental models to study free radical-induced membrane damage or the lipid oxidation in foods containing PUFA and to evaluate antioxidant properties of phenolic compounds (Cirico & Omaye, 2006; Dai et al., 2006; Kerry & Abbey, 1997; Lee et al., 2006).

Mashua (*Tropaeolum tuberosum* Ruíz and Pavón) is an Andean tuber cultivated for centuries in Peru, Bolivia, Ecuador, Venezuela and Colombia. Andean people consuming mashua claim health improvement related to kidney and liver pain, skin eczemas and prostate disorders (Grau, Ortega, Nieto, & Hermann, 2003). Studies performed by our group showed that the purple mashua genotypes have a high content of phenolic compounds, comparable with those found in already known antioxidant sources (Campos et al., 2006). In addition, the purple-coloured mashua tubers presented eight to ten times higher antioxidant capacity than the yellow-coloured tubers (Chirinos et al., 2007a). It has also been reported that phenolic contents and ORAC values ranged, respectively, from 174 to 275 mg gallic acid equivalents (GAE)/100 g fresh matter (FM) (Chirinos et al., 2006) and from 273 to 379 μmol of trolox equivalents (TE)/g dry matter (DM) for the purple-coloured mashua (Chirinos et al., 2007a). These ORAC values are higher than the values reported for various berry species (blackberry, red raspberry, black raspberry, strawberry: 35–162 μmol TE/g DM) (Wang & Lin, 2000). We recently reported that anthocyanins were the major contributors to the *in vitro* ABTS antioxidant capacity of mashua for only one of the three studied purple mashua genotypes, suggesting that other phenolics present are playing a major role in the antioxidant capacity of mashua tubers (Chirinos et al., 2006). Proanthocyanidins, which have been reported to be present in mashua tubers are possibly the antioxidant compounds in that respect (Chirinos et al., 2008). The above mentioned studies encouraged us to study mashua phenolic compounds in terms of their biological properties, and more specifically in terms of *in vitro* antioxidant capacity against oxidation of biological structures rich in PUFA (LDL and erythrocytes).

The goal of this study was first, to evaluate the ability of mashua phenolic extracts to inhibit *in vitro* LDL oxidation, as well as oxidative hemolysis of human isolated red blood cells, and second, to relate these properties to the ORAC values and phenolic composition of the mashua phenolic extracts.

2. Materials and methods

2.1. Samples

Three purple-coloured mashua (*T. tuberosum*) genotypes were chosen from their already demonstrated high hydrophilic antioxidant capacity (Chirinos et al., 2007a): ARB 5241 (peel/flesh, purple/yellow), AGM 5109 (peel/flesh, purple/yellow) and DP 0224 (peel/flesh, purple/purple), together with one yellow-coloured genotype ARB 5576 (peel/flesh, yellow/yellow). Genotypes were kindly provided by the International Potato Center (CIP, Lima, Peru). All mashua tubers were harvested at 7.5 months of maturity stage (full maturity). Harvested tubers were immediately lyophilized and stored at −20 °C. Commercially available powdered polyphenolic extracts, Provinols™ and Prolivols™ (Société Française de Distilleries, France), were purchased from a local market in Belgium.

2.2. Reagents and standards

Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid), fluorescein sodium salt, 2 N Folin–Ciocalteu reagent, potassium bromide (KBr), ethylenediamine tetra-acetic acid (EDTA), tris(hydroxymethyl)-aminomethane (Trizma®Base), 2-thiobarbituric acid (TBA), tricloroacetic acid (TCA), 4-(dimethylamino)-cinnamaldehyde solution (DMACA) and malondialdehyde (MDA) were purchased from Sigma Chemicals Co. (St. Louis, MO) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) from WAKO Chemicals Industries (Orokama, Japan). Gallic and chlorogenic acids and quercetin were purchased from Sigma Chemicals Co. (St. Louis, MO). Catechin was purchased from ChromaDex™ (Santa Clara, CA). Delphinidin-3-glucoside was purchased from Extrasynthèse (Genay, France). Copper sulphate $5H_2O$, HPLC-grade acetonitrile and other solvents and reagents were purchased from Merck (Darmstadt, Germany).

2.3. Sample preparation

Approximately 5.0 g of each lyophilized mashua sample was placed in a screw-cap vial wrapped with aluminium foil and extracted with 300 ml of 0.1% HCl in a solvent mixture (methanol/acetone/water, 45/45/10, v/v/v) (Chirinos, Rogez, Campos, Pedreschi, & Larondelle, 2007b). The mixture was vortexed and flushed with nitrogen for 3 min. The vial was allowed to stand in a water bath at room temperature for 60 min under agitation (200 rpm). Then, the extract was centrifuged at 27,000*g* for 10 min at 4 °C and the supernatant was collected. The above extraction was repeated on the pellet by addition of 150 ml of the same mixture for 15 min. The supernatants were combined and evaporated in a rotary evaporator at 38 °C and the residue was diluted with acidified water (0.01% HCl, v/v). The obtained extract is referred to as crude mashua extract (CME). Prior to phenolic fractionation, 25 ml of CME were passed through a C_{18} Sep-pak cartridge (10 g, 35 cc Waters, Milford, MA) (Chirinos et al., 2006), previously activated with methanol and acidified water (0.01% HCl). Phenolic compounds were adsorbed onto the column matrix. Sugars, acids, and other polar compounds were eluted with 70 ml of acidified water (0.01% HCl). The phenolic fraction was then eluted with 100 ml of acidified methanol (0.01% HCl). Methanol was then removed under vacuum (38 °C) and the residue was re-dissolved in 8 ml of Milli-Q water. This extract is referred to as purified mashua extract (PME).

The powdered commercially available polyphenolic extracts (Provinols and Prolivols) were dissolved in Milli-Q water (5 mg/ml) and filtered (No. 50, Whatman) before carrying out the different assays. Three replicates were carried out for all sample preparations.

2.4. LDL oxidation assay

2.4.1. General

Human plasma was provided by Cliniques du Sud-Luxembourg (Luxemburg). LDL was isolated from human plasma by a discontinuous density gradient centrifugation procedure, as described by

Redgrave, Roberts, and West (1975) and Souza et al. (2008). The isolated LDL fraction was dialyzed against a buffered solution (0.15 M NaCl and 10 mM tris(hydroxymethyl)-aminomethane, pH 7.4) with sodium EDTA (0.1 g/l). The LDL fraction was then kept under nitrogen at 4 °C for a maximum of 15 days. EDTA was removed by a 180 min dialysis against EDTA-free buffered solution before performing the oxidation experiments. The protein content in the LDL fraction was determined by the Lowry protein assay (Lowry, Rosebrough, Lewis Farr, & Randall, 1951). Total cholesterol was determined using a cholesterol assay kit (Merck, Darmstadt, Germany). The evaluation of LDL protection against oxidation was performed by the two methods described below.

2.4.2. Copper-induced oxidation, followed by conjugated dienes assay

The conjugated dienes assay was performed according to the protocol described by Souza et al. (2008) using Cu^{2+} ($CuSO_4$) to induce LDL oxidation. The protective effects of extracts (3, 4 and 5 μM of GAE) and phenolic standards (3–5 μM) against LDL oxidation were evaluated. The studied standards were quercetin, catechin, gallic acid, chlorogenic acid and delphinidin 3-glucoside. These five standards were selected due to their high frequency in commonly consumed vegetables and fruits and because some of them were also found in mashua tubers (Chirinos et al., 2006, 2008). Once the oxidation was initiated the production of conjugated dienes was monitored at 234 nm over a 10 h period. The lag time was calculated as the intercept between the tangent of the absorbance curve during the propagation phase and was expressed in minutes. The inhibition of LDL oxidation was calculated as follows:

$$\%\ \text{Inhibition}_{\text{conjugated dienes}} = \left[1 - \left(\frac{\text{Lag time}_{(\text{No antioxidant})}}{\text{Lag time}_{(\text{With antioxidant})}}\right)\right] * 100$$

2.4.3. AAPH-induced oxidation, followed by thiobarbituric acid reactive substances (TBARS) assay

The TBARS assay was performed, following the protocol described by Souza et al. (2008). LDL oxidation was induced by AAPH and assessed by measuring the TBARS levels, which mainly corresponded to the malondialdehyde (MDA) produced by the oxidation process. The protective effects of extracts (1, 2.5 and 5 μM of GAE), and standards (1–5 μM), against the MDA formation derived from LDL oxidation, were evaluated. The produced MDA was measured at 515 nm ($\lambda_{\text{excitation}}$) and 555 nm ($\lambda_{\text{emission}}$) on a fluorometer (Ascent Fluoroscan, Labsystem, Helsinki, Finland) and was calculated from a MDA standard curve. The inhibition of LDL oxidation was calculated as follows:

$$\%\ \text{Inhibition}_{\text{TBARS}} = \left[1 - \left(\frac{\text{MDA}_{(\text{With antioxidant})}}{\text{MDA}_{(\text{No antioxidant})}}\right)\right] * 100$$

2.5. Erythrocyte hemolysis assay

EDTA-treated tubes (3 ml) were used to collect human blood from healthy volunteers at the Centre de Prévention et de Protection – Médecine du Travail (CESI, Louvain-la-Neuve, Belgium). Erythrocytes were isolated as described by Souza et al. (2008).

The protocol proposed by Souza et al. (2008) was used to evaluate the improved resistance conferred by the polyphenolic preparations (extracts at 1, 2.5 and 5 μM of GAE, and standards at 1, 2.5 and 5 μM) to erythrocytes submitted to free radical attacks induced by AAPH. Hemolysis was followed at 534 nm every 90 s for 3 h at 37 °C. The protection of erythrocytes was deduced from the time required ($T_{0.5}$) for half-hemolysis (50% reduction at $\text{Abs}_{540\,\text{nm}}$) compared to control values (phosphate buffer). The inhibition of erythrocyte hemolysis was calculated as follows:

$$\%\ \text{Inhibition}_{\text{Hemolysis}} = \left[1 - \left(\frac{T_{0.5(\text{No antioxidant})}}{T_{0.5(\text{With antioxidant})}}\right)\right] * 100$$

2.6. Oxygen radical absorbance capacity (ORAC) assay

The ORAC analyses in mashua extracts were performed in a 96-well microplate fluorometer and were adapted from the procedures described by Ou, Hampsch-Woodill, and Prior (2001) and Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002). AAPH was used as a peroxyl radical generator, trolox as standard and fluorescein as fluorescent probe. Briefly, 25 μl of blank, trolox standard or diluted sample were mixed with 250 μl of 55 nM fluorescein and incubated for 10 min at 37 °C before automatic injection of 25 μl of AAPH solution (153 nM). Fluorescence was measured at 485 nm ($\lambda_{\text{excitation}}$) and at 520 nm ($\lambda_{\text{emission}}$) every min for 50 min. The final ORAC values were calculated by using the area under the decay curves and were expressed as μmol of TE/100 g of mashua fresh matter (FM) or μmol of TE/g of extract DM.

2.7. Determination of total phenolics (TP), total anthocyanins (TA) and total flavanoids (TFA)

TP contents in mashua extracts were determined with the Folin–Ciocalteu reagent by the method of Singleton and Rossi (1965), using gallic acid as a standard. Absorbance was measured at 755 nm and the results were expressed as mg GAE/100 g mashua FM or mg GAE/g of extract DM. TA were measured using the pH differential method (Giusti & Wrolstad, 2001). Absorbance was measured at 520 and 700 nm in pH 1.0 and 4.5 buffers. A molar extinction coefficient of 26,900 $\text{L cm}^{-1}\ \text{mol}^{-1}$ and a molecular weight of 449.2 were used for anthocyanin calculation. Results were expressed as mg of cyanidin 3-glucoside equivalents (CGE)/g of extract DM. TFA were estimated in the mashua extracts using the chromogen DMACA reagent, following the method proposed by Delcour and Janssens de Varebeke (1985), using catechin as standard. Absorbance was measured at 640 nm and results were expressed as mg of catechin equivalents (CE)/g of extract DM.

2.8. Statistical analysis

Results are reported as means ± SD. Correlation analyses were performed using linear regression and the Pearson's correlation coefficient (r). The SPSS software for Windows 14.0 (SPSS, Chicago, IL) was used to run statistical analyses.

3. Results and discussion

3.1. Total phenolics, total anthocyanins, total flavanoids and ORAC assay

The TP and ORAC values for mashua tubers were 323, 205, 166 and 56.3 mg GAE/100 g FM and 51.8, 32.8, 19.5 and 9.2 μmol TE/g FM, respectively, for the ARB 5241, DP 0224, AGM 5109 and ARB 5576 mashua genotypes (humidity values of 83.4%, 84.2%, 87.0% and 87.1%, respectively). These results are in accordance with previous values reported by our research group for the same mashua genotypes but at different harvest years (Chirinos et al., 2006, 2007b). This is an indication that mashua genotypes maintain their TP contents and ORAC values during different harvest years. TP, TA, TFA and ORAC values for the CME of the four evaluated mashua genotypes ranged from 10.9 to 57.4 mg GAE/g DM, 11.4 to 13.6 mg CGE/g DM, 1.8 to 7.8 mg CE/g DM and 165 to 601 μmol TE/g DM, respectively (Table 1). The clean up procedure with a C_{18} Sep-pak cartridge allowed considerable increases of TP, TA, TFA and ORAC values. Thus, the corresponding PME ranged from

Table 1
Total phenolics (TP), total anthocyanins (TA), total flavanoids (TFA) and ORAC values of crude and purified mashua extracts for the four evaluated mashua genotypes[a]

Mashua genotype	TP (mg GAE/g extract DM)[b]	TA (mg CGE/g extract DM)[c]	TFA (mg CE/g extract DM)[d]	ORAC (μmol TE/g extract DM)[e]
CME				
ARB 5241$^{(p/y)}$	57.4 ± 3.8	11.4 ± 1.1	7.8 ± 0.4	601 ± 30
AGM 5109$^{(p/y)}$	30.1 ± 1.6	5.9 ± 0.2	4.5 ± 0.2	335 ± 19
DP 0224$^{(p/p)}$	40.8 ± 4.9	13.6 ± 0.9	4.3 ± 0.3	447 ± 41
ARB 5576$^{(y/y)}$	10.9 ± 2.3	–	1.8 ± 0.3	165 ± 8[d]
PME				
ARB 5241$^{(p/y)}$	728 ± 7.7	165 ± 10.2	112 ± 12.3	7801 ± 666
AGM 5109$^{(p/y)}$	645 ± 5.1	103 ± 11.1	71.9 ± 8.2	5903 ± 567
DP 0224$^{(p/p)}$	441 ± 34.4	187 ± 9.7	63.8 ± 3.9	4873 ± 398
ARB 5576$^{(y/y)}$	209 ± 18.8		22.3 ± 2.3	4147 ± 698

(p/y) purple peel/ yellow flesh, (p/p) purple peel/ purple flesh, (y/y) yellow peel/ yellow flesh. CME: crude mashua extract; PME: purified mashua extract.
[a] Data are expressed as means ± SD of three replicates.
[b] Total phenolics expressed as mg of gallic acid equivalents/g extract DM (GAE/g DM).
[c] Total anthocyanin content expressed as mg of cyanidin 3-glucoside equivalents/g extract DM (CGE/g DM).
[d] Total flavanoids expressed as mg of catechin equivalents/g extract DM (CE/g DM).
[e] Antioxidant capacity expressed as μmol of trolox equivalents/g extract DM (TE/g DM).

209 to 728 mg GAE/g DM, 165 to 187 mg CGE/g DM, 22.3 to 112 mg CE/g DM and 4147 to 7801 μmol TE/g DM, respectively (Table 1). The increases of these characteristics in the mashua extracts after SPE treatment may be attributed in part to the elimination of water-soluble constituents (e.g., sugars, ascorbic acid, aminoacids). Calculation of the purification rate between PME and CME for TP, TA and TFA, yielded values that ranged from ~10 to 21, ~13 to 17 and ~12 to 16, respectively.

TA in the PME represented 15.9%, 22.7% and 42.4% of the TP for AGM 5109, ARB 5241 and DP 0224, whereas TFA represented 10.6%, 11.1%, 14.4% and 15.3% of the TP for ARB 5576, AGM 5109, DP 0224 and ARB 5241, respectively (Table 1). These percentages suggested that other important families of phenolic compounds are contained in the PME (especially for the ARB 5576 yellow genotype) or that the degree of polymerization of the polyphenols present in the extracts is high (Souza et al., 2008). Souza et al. (2008) mentioned that the polymerization degree of polyphenols can be estimated by the ratio between the TP and TFA contents. Taking this concept into account, PME for ARB 5241, DP 0224, AGM 5109 and ARB 5576 presented polymerization degrees of 6.5, 6.9, 8.9 and 9.3, respectively. Because PME presented high concentrations of TP, TA and TFA (mg/g DM) than did CME, it was decided to evaluate the antioxidant capacity of these extracts against oxidative damage caused by lipid oxidation on biological structures (LDL and erythrocytes).

3.2. Evaluation of the antioxidant capacity of mashua extracts through biological in vitro assays

The role of phenolics from mashua in exerting antioxidant protective effects on biological structures, such as LDL and erythrocytes has not yet been studied. Thus, to evaluate this property, three biological *in vitro* assays were used. Two of them evaluated the protection conferred by the extracts to LDL subjected to oxidative damage, using two different inductors of oxidation: the azo compound AAPH (TBARS assay) and Cu^{2+} (conjugated dienes assay). The third assay evaluated the ability of mashua extracts to delay the AAPH-induced hemolysis of erythrocytes. As detailed above, all these assays are based in an attack on the PUFA of the biological structures, using different oxidative systems to measure the inhibition of lipid oxidation given by an antioxidant compound.

All PME presented biological protective effects against LDL and erythrocyte oxidation (Fig. 1). A dose-dependent inhibition against human LDL oxidation, for both TBARS and conjugated dienes assays (Fig. 1a and b, respectively), as well as for the oxidative hemolysis of erythrocytes (Fig. 1c), was found. Similar dose-dependent inhibition effects against LDL oxidation and hemolysis of erythrocytes have been reported in extracts from various products (Lee et al., 2006; Viana et al., 1996; Zhu et al., 2002). For the three evaluated biological oxidative assays, similar inhibition values were found for the four PME for the investigated range of phenolic concentrations (Fig. 1). The highest values of inhibition against LDL and erythrocytes oxidation for PME were obtained at 5 μM GAE. At this concentration, the inhibition of LDL oxidation for the PME ranged from 29.1% to 34.8% and from 51.8% to 58.1%, respectively, for the AAPH-induced (TBARS assay) and copper-induced (conjugated dienes assay) oxidation assays (Fig. 1a and b), and from 20.8% to 25.1% for the AAPH-induced hemolysis of erythrocytes (Fig. 1c).

To know the level of PME anti-oxidative potential, it was decided to compare the above obtained results for the three biological *in vitro* assays with those displayed by the commercially available antioxidant polyphenolics: Provinols™ (an alcohol-free red wine extract) and Prolivols™ (a product obtained from the aqueous fraction of the olive fruit using a physical extraction process, Société Française de Distilleries, 2007). Provinols™ and Prolivols™ also displayed good inhibitory effects against LDL oxidation for both TBARS and conjugated dienes assays (Fig. 1a and b, respectively) as well as for the oxidative hemolysis of erythrocytes (Fig. 1c), showing a dose-dependent inhibitory trend. At 5 μM GAE, the inhibitions of LDL oxidation for the Provinols™ and Prolivols™ were 40.6% and 22.6% for TBARS assay and 69.2% and 64.4% for conjugated dienes assay (Fig. 1a and b), respectively, whereas the inhibitions against oxidative hemolysis of erythrocytes were 24.9% and 100%, respectively (Fig. 1c). In terms of inhibition against LDL oxidation, Provinols™ and Prolivols™, for the evaluated phenolic concentrations, presented values close to those found for the PME (Fig. 1a and b, respectively). By contrast, Prolivols™ showed a better protective effect against oxidative inhibition of erythrocytes than did PME and Provinols™ (Fig. 1c).

The protection rates of PME, Provinols™ and Prolivols™ against LDL and erythrocyte oxidation might be due to different mechanisms. First, the partition coefficient or degree of lipophilicity of phenolic compounds (Liao & Yin, 2000) determines their interaction with different biomembranes and influences their antioxidant capacity performance. On the other hand, some polyphenols that do not interact with biomembranes, such as flavanoids, have been reported to display major interactions with free radicals present in the aqueous phase of the oxidation system (Liao & Yin, 2000). Second, the chemical structure of the phenolic compounds determines their ability to react with free radicals. The radical-scavenging capability of flavonoids has been related to three structural groups:

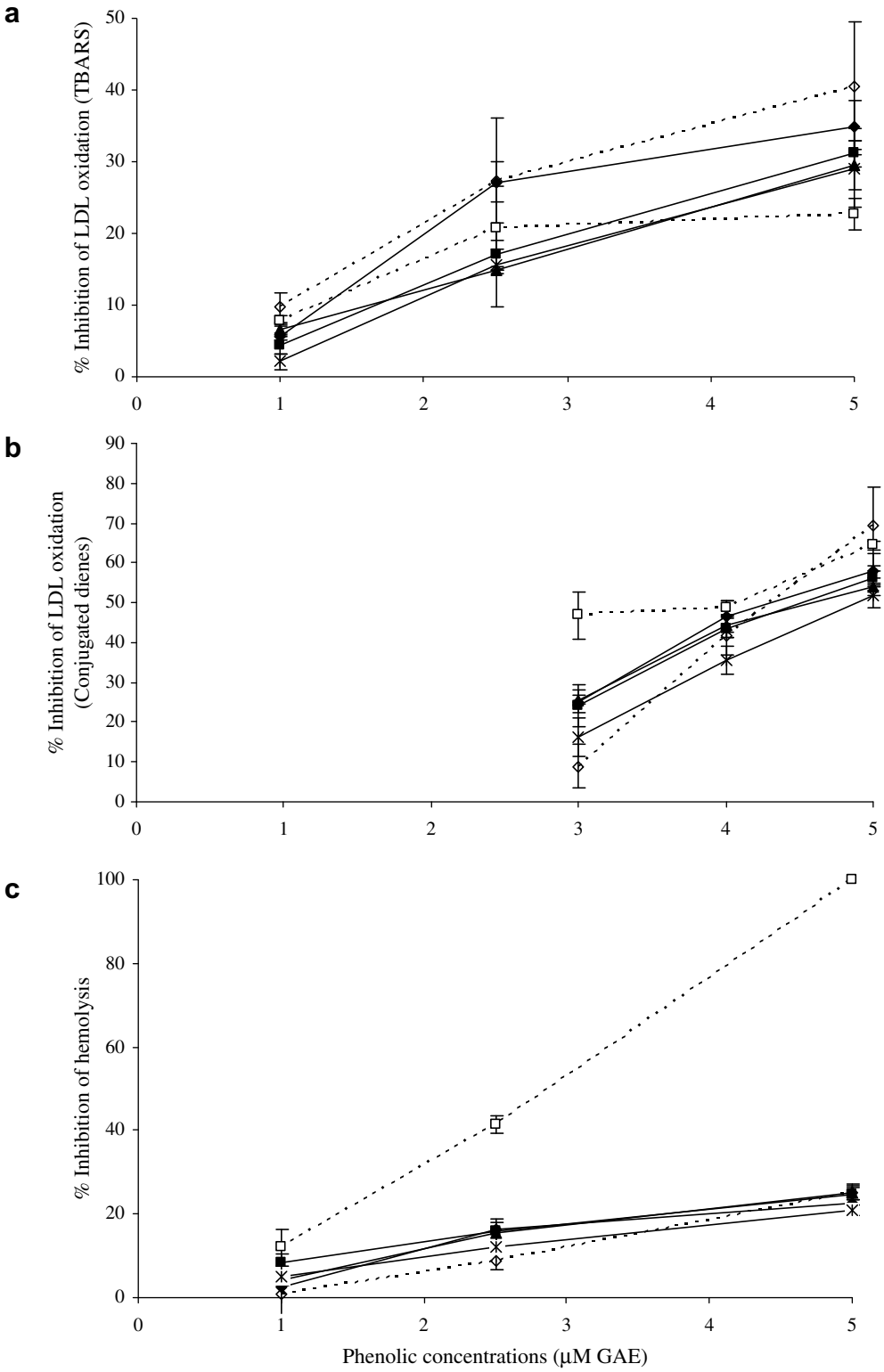

Fig. 1. Protective effects of purified mashua extracts (PME) from four mashua genotypes: ARB 5241 (-♦-), DP 0224 (-■-), AGM 5109 (-▲-), ARB 5576 (-×-), Provinols™ (–◇–) and Prolivols™ (–□–) on (a) human LDL subjected to AAPH-induced oxidation (TBARS assay), (b) human LDL subjected to copper-induced oxidation (conjugated dienes assay) and (c) human erythrocytes subjected to AAPH, as a function of the phenolic concentrations of the extracts (expressed as μM GAE). Bars indicate mean values ± SD for three replicates.

the *o*-dihydroxyl structure of the B ring, the 2,3-double bond, in conjunction with the 4-oxo function, and the additional presence of both 3-and 5-hydroxyl groups (Vaya et al., 2003). For phenolic acids, the free radical-scavenging properties depend mainly on their numbers of hydroxyl groups (Hsieh, Yen, & Chen, 2005). Third, the antioxidant effect of phenolics is concentration-dependent. Phenolics could present good antioxidant effects among a range of concentrations, as demonstrated above, but it is possible

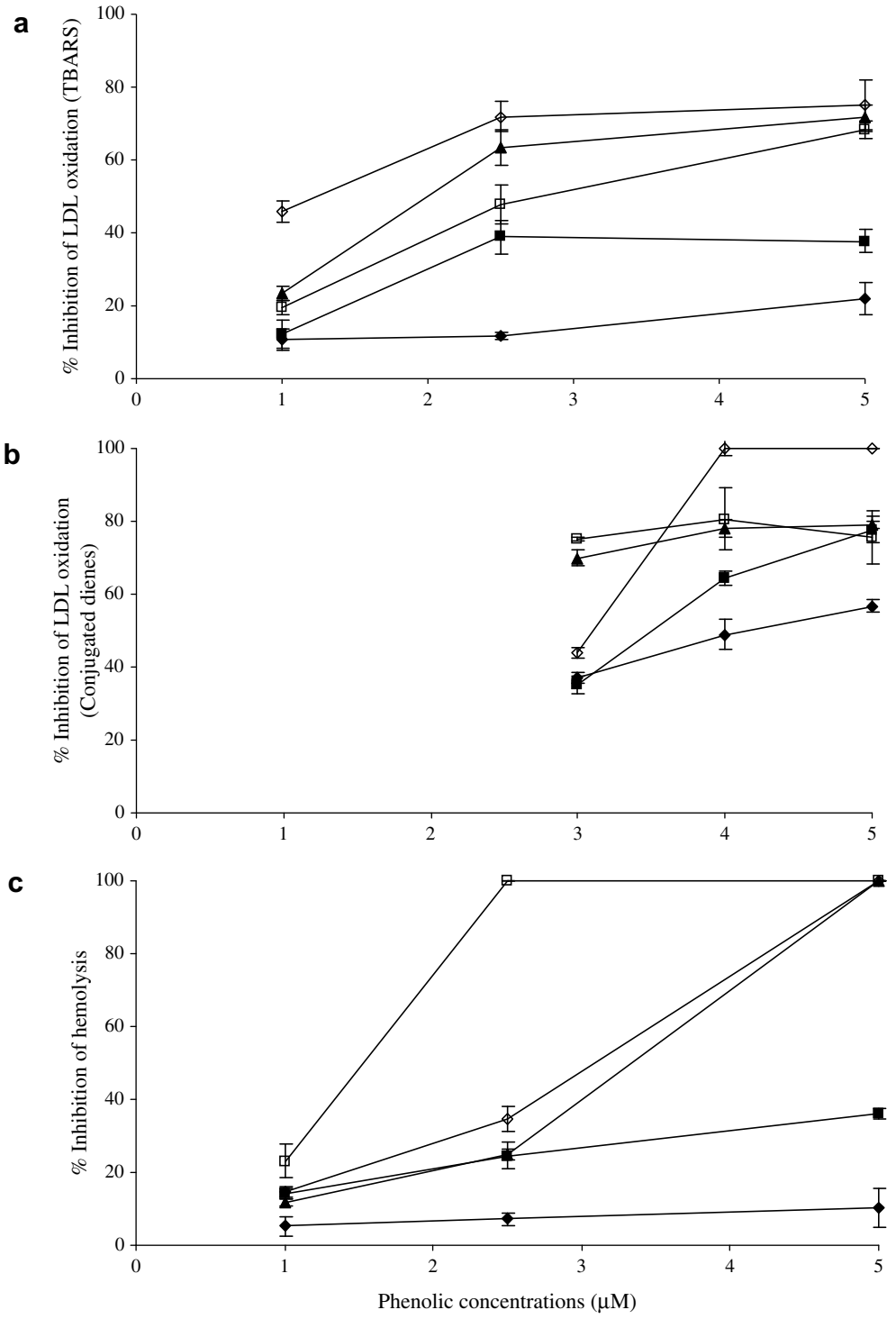

Fig. 2. Protective effects of phenolic standards on (a) human LDL subjected to AAPH-induced oxidation (TBARS assay), (b) human LDL subjected to copper-induced oxidation (conjugated dienes assay) and (c) human erythrocytes subjected to AAPH, as a function of the phenolic concentrations of the standards (expressed as μM) tested. Gallic acid (-♦-), chlorogenic acid (-■-), catechin (-▲-), quercetin (-◇-) and delphinidin-3-glucoside (-□-). Bars indicate mean values ± SD for three replicates.

that pro-oxidant effects could appear at certain concentrations (Cirico & Omaye, 2006). Four, in phenolic mixtures (e.g., PME, Provinols™ and Prolivols™), it is possible that the presence of synergistic, additive or antagonistic effects modifies the inhibitory characteristics of the samples. Finally, the results of the determination of the antioxidant capacity of an extract depend greatly on the methodology used in terms of the oxidant and the oxidable substrate (Souza et al., 2008; Zhu et al., 2002).

Considering that the capacity to protect LDL and erythrocytes against oxidation is largely related to the different characteristics of the phenolic compounds present in the extracts, it is important to identify and quantify the different polyphenolic compounds present in these extracts. The presence of flavan-3-ols (gallocatechin, epigallocatechin, epicatechin and its derivatives), procyanidin B_2, hydroxycinnamic (derivatives of *o*-coumaric and *p*-coumaric acid) and hydroxybenzoic acids (derivatives of gallic, hydroxybenzoic

Table 2
Pearson's correlation coefficients (r) among the analyzed parameters

	TP	TA	TFA	ORAC	LDL-AAPH (TBARS assay)	LDL-Cu^{2+} (conjugated dienes assay)
TP	1					
TA	−0.228	1				
TFA	0.894**	0.012	1			
ORAC	0.854**	0.007	0.914**	1		
LDL-AAPH (TBARS assay)	0.645*	0.290	0.907**	0.781*	1	
LDL-Cu^{2+} (conjugated dienes assay)	0.584*	0.557*	0.837*	0.642*	0.960**	1
Hemolysis	0.276	−0.137	0.143	0.023	0.148	0.064

* Correlations significant at a 0.05 level (1-tailed).
** Correlations significant at a 0.01 level (1-tailed).

and protocatechuic acid) and proanthocyanidins in all PME (Chirinos et al., 2008), as well as of the anthocyanins (mainly delphinidins) in the purple purified mashua extracts (Chirinos et al., 2006), has recently been reported. Proanthocyanidins in mashua tubers are one of the major contributors to their antioxidant capacity (Chirinos et al., 2008). On the other hand, Provinols™ contains, as representative phenolic compounds: proanthocyanidins, anthocyanins, catechin (flavan-3-ol), hydroxycinnamic and flavanols (480, 61, 38, 18 and 14 mg/g of DM, respectively) (Zenebe, Pecháňová, & Andriantsitohaina, 2003). Thus the four PME and Provinols™ have, as common phenolic families, the flavan-3-ols and proanthocyanidins. Possibly, due to the representative participation of these phenolic families in the extracts, close values of lipid oxidation inhibition are reached for the three evaluated biological *in vitro* assays. Other studies have shown protective effects of the phenolic families mentioned above. Thus, phenolic fractions from red wine, composed of flavan-3-ol compounds and phenolic acids, showed good protective effects against AAPH-induced and copper-induced LDL oxidation (Kerry & Abbey, 1997). Tedesco et al. (2000) reported that polyphenols of red wine, such as procyanidins, reduce the oxidation of erythrocytes. In addition, flavan-3-ols with a gallate group, such as gallocatechin, epigallocatechin, gallocatechin gallate, have been shown to inhibit copper-induced plasma oxidation (Hashimoto, Yaika, Tanaka, Hara, & Kojo, 2000). Prolivols™ is an extract with a quite different composition. It is rich in hydroxytyrosol (a phenylethyl alcohol) (Société Française de Distilleries, 2007). This compound, as well as other phenolic compounds contained in this extract, offers similar values of inhibition against LDL oxidation as PME and Provinols™, but is more efficient in inhibiting hemolysis of erythrocytes. Interestingly, Manna, Galletti, Cucciolla, Montedoro, and Zappia (1999) found that olive oil hydroxytyrosol efficiently protected human erythrocytes against oxidative damages induced by hydrogen peroxide.

When the protective effects of phenolic standards at different phenolic concentrations were evaluated using the three biological *in vitro* assays, we observed that the standards displayed high antioxidant effects against oxidation of LDL and erythrocytes (Fig. 2). The standards that belong to the flavonoid family (quercetin, catechin and delphinidin-3-glucoside) presented higher inhibition values than did the phenolic acids. At 5 μM, quercetin drastically inhibited (100%) the oxidation of LDL in the conjugated dienes assay and delphinidin 3-glucoside, quercetin and catechin showed a 100% inhibition against oxidative hemolysis of erythrocytes. Catechin, quercetin, and delphinidin-3-glucoside inhibited the oxidation of LDL induced by AAPH but only by around 70%. These results confirm those reported in previous studies regarding the protective effects of anthocyanins (Kähkönen & Heinonen, 2003), quercetin and catechin (Liao & Yin, 2000; Vaya et al., 2003) in terms of human LDL oxidation and oxidative hemolysis. Flavonoid standards showed greater values of inhibition of LDL oxidation than did PME, Provinols™ and Prolivols™, for both evaluated assays. Prolivols™ displayed values of inhibition against oxidative hemolysis similar to quercetin and catechin standards. In general, phenolic acid standards presented lower inhibitions against oxidation of LDL and erythrocytes than did the flavonoids. This result is probably due to their anionic forms at pH 7.4 and consequently they are very soluble in the aqueous phase of the systems and present very low affinities for biomembranes (also charged negatively).

The results found suggest that phenolic compounds are substances that present important inhibiting roles against lipid oxidation of biological molecules. Thus, phenolics could be applied on biological molecules or in lipid food matrices to inhibit oxidative damage, opening the door to a large number of applications in food and cosmetic industries.

3.3. Correlations between antioxidant properties and phenolic compounds

Finally, correlations were tested to link the different analyzed parameters during this study (Table 2). High correlations were found between ORAC values on the one hand and TP or TFA on the other. This is in accordance with other studies (Souza et al., 2008; Wang & Lin, 2000). Also, good correlations were found for protection against AAPH-induced and Cu^{2+}-induced LDL oxidation and TP, TFA and ORAC. Souza et al. (2008) also found good correlations among the mentioned assays using the same methodology. A good correlation between TA and Cu^{2+}-induced LDL oxidation was found. Inhibition of hemolysis was not correlated with TP and TFA but a negative correlation with TA was found. These results suggest that most of these compounds have a low or limited ability to protect lipid targets in the erythrocyte membrane. Furthermore, hemolysis did not show good correlations with the two LDL oxidation assays.

4. Conclusions

In conclusion, the results show that, *in vitro*, PME can inhibit LDL oxidation in both a radical-induced (AAPH) and a metal-induced (Cu^{2+}) system. Inhibition of AAPH-induced oxidative hemolysis of erythrocytes was also found. These results indicate that mashua phenolics are capable of scavenging peroxyl radicals, as well as chelating redox metal ions *in vitro*. We presume that these protective effects of mashua extracts are dependent on their phenolic composition in terms of lipophilicity/hydrophilicity partition, and molecular structures that define their reactivity against free radicals, as well as on the phenolic concentrations. Possibly, synergistic, additive or antagonistic actions are also present. Finally, the present study suggests that phenolic compounds of mashua provide a good source of dietary antioxidants that could offer potential protective effects against lipid oxidation and which could be exploited by the food or cosmetics industry.

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