Plant Foods Hum Nutr (2015) 70:371–379
DOI 10.1007/s11130-015-0516-3

ORIGINAL PAPER

Effects of the Dietary Addition of Amaranth (*Amaranthus mantegazzianus*) Protein Isolate on Antioxidant Status, Lipid Profiles and Blood Pressure of Rats

María B. Lado[1] · **Julieta Burini**[1] · **Gustavo Rinaldi**[2] · **María C. Añón**[1] · **Valeria A. Tironi**[1]

Published online: 24 October 2015

Abstract The effects of the dietary addition of 2.5 % (*w*/*w*) *Amaranthus mantegazzianus* protein isolate (AI) on blood pressure, lipid profiles and antioxidative status of Wistar rats were evaluated. Six diets were used to feed animals during 28 days: (base (AIN93G), Chol (cholesterol 1 %, *w*/*w*), CE (α-tocopherol 0.005 %, *w*/*w*), CholE (cholesterol 1 % (*w*/*w*) + α-tocopherol 0.005 %, *w*/*w*), CAI (AI 2.5 % *w*/*w*), CholAI (cholesterol 1 % (*w*/*w*) + AI 2.5 %, *w*/*w*). Lipid profiles of plasma and liver and faecal cholesterol content were analyzed. Antioxidant status was evaluated by the ferric reducing activity of plasma (FRAP), the 2-thiobarbituric acid (TBA) assay and superoxide dismutase (SOD) activity in plasma and liver. Blood pressure was measured in the tail artery of rats. CholA group presented a significant ($\alpha < 0.05$) reduction (16 %) in the plasma total cholesterol. In liver, the intake of cholesterol (Chol group) induced a significant increment in cholesterol and triglycerides (2.5 and 2.3 times, respectively), which could be decreased (18 % and 47 %, respectively) by the addition of AI (CholA group). This last group also showed an increased faecal cholesterol excretion (20 %). Increment (50 %) in FRAP values, diminution of TBA value in plasma and liver (70 % and 38 %, respectively) and diminution of SOD activity (20 %) in plasma of CholA group suggest an antioxidant effect because of the intake of AI. In addition, CA and CholA groups presented a diminution (18 %) of blood pressure after 28 days.

Keywords Amaranth protein isolate · Wistar rats · Antioxidant status · Hypolipidemic profile · Hypotensive effect

✉ Valeria A. Tironi
vtironi@quimica.unlp.edu.ar; http://www.nepsy.szote.u-szeged.hu/~kincsesz

[1] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) – CCT La Plata-CONICET, UNLP, 47 y 116 (1900), La Plata, Argentina

[2] Centro de Investigaciones Cardiovasculares - CCT La Plata-CONICET, Facultad de Medicina (UNLP), 60 y 120 (1900), La Plata, Argentina

Introduction

Amaranth (Amaranthaceae) is an ancestral American pseudocereal with excellent agroecological behavior and interesting nutritional quality, presenting high protein content (15–17 %, *w*/*w*) and good amino acid balance. In addition, there is currently a strong interest in amaranth seeds due to their potential physiological effects. Amaranth proteins and peptides have demonstrated diverse biological activities. Among them, inhibitory action against the angiotensin converting enzyme (ACE) by peptides has been proved [1] while Fritz et al. [2] demonstrated an *in vivo* hypotensive effect of hydrolysates in hypertensive rats. Moreover, a significant reduction of the *in vitro* micellar solubility of cholesterol due to protein hydrolysates has been observed [unpublished work]. Hypocholesterolemic, hypotriglyceridemic and antioxidant effects of an amaranth protein concentrate in Wistar rats [3] and hypocholesterolemic effect of protein isolate in hamsters [4] have been informed for *A. cruentus*. Antioxidant properties of amaranth have been attributed to polyphenolic compounds and squalene [5, 6]. However, amaranth proteins have also demonstrated by *in vitro* experiments to be a potential source of antioxidant peptides with action on different reactive species, which could be released into the human body after gastrointestinal digestion [7, 8]. Taking account of the antecedents related to the potential biological actions of amaranth peptides, in the present work we proposed to explore *in vivo*

beneficial effects of the intake of an *A. manteggazianus* protein isolate, focusing on its action on blood pressure, the lipid profiles and the antioxidative status.

Materials and Methods

Protein Isolate (AI) *A. mantegazzianus* (*Pass. cv Don Juan*) was grown at Facultad de Agronomía, Universidad Nacional de La Pampa (Argentina) and at Facultad de Agronomía y Veterinaria, Universidad Nacional de Rio Cuarto, Córdoba (Argentina). Flour was obtained by grinding the whole seeds in an Udy mill, 1 mm mesh, screened by 0.092 mm mesh, and defatted with hexane (24 h, 4 °C). AI was obtained from the defatted flour by extraction at pH 9, isoelectric precipitation (pH = 5), neutralization and freeze-drying [9]. AI contained 78 ± 1 g proteins/100 g, 10 ± 1 g water/100 g, and 2.8 ± 0.1 g ash/100 g, 1.1 ± 0.1 g lipids/100 g, 8.2 ± 0.2 g carbohydrates/100 g.

Diets Preparation Six isocaloric and isoprotein formulations were prepared using the AIN-93G [10] as basal diet: basal, Chol (cholesterol (chol) 1 %, *w/w*), CE (α-tocopherol (E) 0.005 %, *w/w*), CholE (chol 1 % (*w/w*) + E 0.005 %, *w/w*), CAI (AI 2.5 %, *w/w*), CholAI (chol 1 % (*w/w*) + AI 2.5 %, *w/w*) (Table 1). Dried ingredients were mixed in a blade mixer during 2 min.; liquid ingredients were added, homogenized during 15 min. The powders were tightly packed and stored at 4 °C.

Animal Assays Wistar rats were obtained from the Comisión Nacional de Energía Atómica (CNEA) (Centro Atómico Ezeiza, Argentina), and maintained at the Facultad de Ciencias Médicas (UNLP) with all the experimental procedures supervised and approved by the Ethics Committee of the institution. 42 male rats (300–400 g) were distributed into six groups, each one fed with a specific diet: ***C*** (basal diet), ***CE*** (CE diet), ***CAI*** (CAI diet), ***Chol*** (Chol diet), ***CholE*** (CholE diet), ***CholAI*** (CholAI diet). The rats were housed in stainless steel cages (3 rats/cage) with sterilized bedding (wood shavings) which was changed every day. They had air conditioning and a 12 h light/12 h dark cycle. Feeding and drinking water were *ad libitum*; tap water was provided in sterilized bottles with stainless steel nipples. All groups had one adaptation week consuming basal diet. After that, each group was fed with its specific diet during four weeks. Body weight gain and food intake were weekly controlled. During the last three days of assay, rats from ***Chol*** and ***CholA*** groups were placed in independent metabolic cages to collect faeces and urine. These samples were daily collected, tightly packed and stored at −80 °C. After four weeks of specific feeding, rats were sacrificed in order to obtain the blood and liver samples. Rats fasted for at

Table 1 Composition of the diets

Ingredient (g/kg)	C	CE	CAI	Chol	CholE	CholAI
Casein[1]	200.00	200.00	178.50	200.00	200.00	178.50
Dextrin[2]	629.50	629.45	626.00	619.50	619.45	616.00
Soy oil[3]	70	70	70	70	70	70
Fiber[4]	50	50	50	50	50	50
Choline[5]	2.5	2.5	2.5	2.5	2.5	2.5
L-Cystine[6]	3	3	3	3	3	3
Mix of vitamins[7]	10	10	10	10	10	10
Mix of minerals[8]	35	35	35	35	35	35
α-Tocopherol (E)[9]	–	0.05	–	–	0.05	–
Cholesterol (chol)[10]	–	–	–	10	10	10
Amaranth protein isolate (AI)	–	–	25	–	–	25

[1] Milk casein, 92 % (*w/w*) protein (Droguería Industrial Lanús S.A., Argentina)

[2] Dextrin (COFEM S.A., Argentina)

[3] Antioxidants-free soy oil (Molinos Río de La Plata, Argentina)

[4] Microcrystaline celullose 20 μm (SIGMA)

[5] Crystaline choline cloride (SIGMA)

[6] L-cystine >99 % (ANEDRA)

[7] α-tocopherol acetate (SIGMA)

[8] according to AIN-93-VX [12]

[9] according to AIN-93G-MX [12]

[10] Cholesterol (SIGMA)

least 14 h were anesthetized by intraperitoneal injection (sodium pentobarbital 25 mg/kg and diazepam 2.5 mg/kg), and then injected with 0.05 mL of heparine (5000 U/mL). Blood was collected by a fine catheter inserted in the abdominal aorta using heparine as anticoagulant. After full collection of blood, rat body was perfused with saline solution with heparine (50 U/mL) using a peristaltic pump (flow =13.5 mL/min) in order to eliminate all the blood in the tissues. After that, liver was dissected. Blood was immediately centrifuged (1000 × *g*, 10 min, room temperature) in order to separate the plasma. A portion of plasma was immediately frozen in liquid nitrogen and then stored at −80 °C; the other portion was stored at 4 °C and used for lipid profile determinations. Liver was immediately frozen in liquid nitrogen and stored at −80 °C.

Blood Pressure Measurements Blood pressure was measured in the tail artery of rats. Rats were placed in a chamber at 37 °C for 10 min and then transferred to a standard setup with heating pad and acrylic restrainer, tail cuff and pulse sensor (Narco Biosystems, Houston, USA). The tail cuff was connected to a cylinder of compressed air through an arrangement of inlet and outlet valves that permitted inflation and deflation of the cuff at a constant rate. Tail cuff pressure was continuously recorded with a solid state pressure sensor (Sensym, All Sensors, Inc., USA). The signals from the pulse and pressure sensors were conveniently amplified and then digitized with an analogue–digital board (DT16EZ, Data Translation, Inc., USA). On-line control display and data were obtained with appropriate software (Labtech Notebook Pro, Laboratory Technology Corp., USA). Measurements (3 for each rat) at the beginning (day 1), at the middle (day 14) and at the end (day 28) of the assay period were performed.

Plasma Lipid Profile Determinations Commercial kits were used: HDL-cholesterol (precipitating reactive (dextran sulphate) and total cholesterol (Enzymatic Colestat, Wiener, Laboratorios S.A.I.C. Rosario, Argentina), and triglycerides (TG Color GPO/PAP AA, Wiener). LDL-cholesterol was calculated using the Friedewald's equation [11]: *LDL-Cholesterol = Total Cholesterol – HDL-Cholesterol -* (*Triglycerides*/*5*).

Determinations of Liver Lipids Liver (250 mg) was homogenized with 5 mL of a chloroform/methanol (2:1) mixture. After 40 min, 1 mL of water was added and, after homogenization of the mixture, it was centrifuged (3000 × *g*, 10 min, room temperature). Aqueous phase was discarded, chloroform phase was filtered and the solvent evaporated, obtaining total lipids by weighing [12]. Lipids were suspended in a 5 g/L Tritón X-100 solution. Total cholesterol and triglycerides were determined using the commercial kits.

Faecal Cholesterol Neutral cholesterol was extracted. Freeze-dried faeces (0.2 g) were mixed with 2 mL of a 10 mol/L NaOH/96 % ethanol (1:2 *v*/*v*) mixture. Suspensions were homogenized and incubated at 70 °C during 45 min. After cooling at room temperature, they were centrifuged (5 min, 1000 × *g*, room temperature). The supernatant was extracted twice with 1 mL hexane. Hexane phases were mixed and washed with 70 % (*v*/*v*) ethanol up to neutrality and hexane was evaporated [13]. Cholesterol was suspended in the mobile phase acetonitrile/isopropanol (70:30) mixture (75 min, 600 rpm, 23 °C) and analyzed by RP-HPLC using a 5 μm C18 Symmetry column (150 × 4.4 mm I.D.), flow: 1 mL/min, temperature: 35 °C, detection at 210 nm. A calibration curve with pure cholesterol solutions (0.1–0.5 mg/mL) was obtained [14].

Antioxidant Capacity of Plasma Three different methodologies were applied:

Ferric reducing antioxidant power (FRAP): 200 μL of FRAP reactive (1 volume of 10 mM 2,4,6-Tripyridyl-s-Triazine (TPTZ) in 40 mM HCl + 1 volume 20 mM $FeCl_3.6H_2O$ + 10 volumes buffer 0.3 M acetate, pH: 3.6) were incubated during 5 min at 37 °C and absorbance at 593 nm (Synergy HT, BioTek, USA) was measured. Sample (7 μL) and water (20 μL) were added. After 8 min of agitation at 37 °C, absorbance at 593 nm was measured. Calibration curve was prepared with $Fe_2SO_4.7H_2O$ solutions (25–250 μmol/L). FRAP value was expressed as mmol Fe^{+2}/L plasma [15].

2-Thiobarbituric acid (TBA) assay. Plasma (100 μL) was treated with 2 mL 0.08 N H_2SO_4 and 250 μL of 10 % (*w*/*v*) phosphotungstic acid. After 5 min, mixtures were centrifuged (1600 × *g*, 10 min, room temperature), pellets were treated with 1 mL 0.08 N H_2SO_4 and 150 μL 10 % (*w*/*v*) phosphotungstic acid, centrifuged, suspended in 2 mL pure water, mixed with 500 μL 0.7 % (*w*/*v*) TBA and incubated at 95 °C during 60 min. After cooling, 2.5 mL of butanol were added and agitated vigorously. Butanol phase was separated (1600 × *g*, 10 min, room temperature) and its fluorescence (λ_{exc} = 485 nm, λ_{em} = 528 nm) was read (Synergy HT, BioTek). A 125 nmol/L 1,1,3,3 tetraethoxypropane solution was used as standard [16].

Superoxide dismutase (SOD) activity. A commercial kit (19,160 SOD determination kit, SIGMA, USA) based on the reaction of a tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrofenyl)-5-(2,4-disulphophenyl)-2 H) with the superoxide anions (generated by xanthine oxidase XO) producing the colour compound WST-1 formazan was used. Mixtures of samples (4 μL), water (16 μL), WST-1 (200 μL) and XO (20 μL) were incubated during 30 min at 37 °C with agitation, reading the OD

at 440 nm every 5 min. Results were expressed as: SOD activity (%) = $(A_B - A_M) \times 100/A_B$, where A_B = OD of blank and A_M = OD of sample.

Antioxidant Capacity of Liver Liver homogenates were prepared from 0.2–0.25 g of liver and 2 mL of cold phosphate-buffered saline (PBS), buffered by homogenization in an ice bath with an Ultraturrax T8.01 (IKA, Germany) equipment (2 min, 20,000 rpm) and centrifugation (10,000 × *g*, 5 min, 4 °C). The supernatant was separated and stored at 4 °C. *TBA assay* and *SOD activity* were measured as previously explained.

Statistical Analysis

Data were analyzed by means of analysis of variance (ANOVA), using a Microsoft Office Excel 97–2003 software. When significant differences ($\alpha = 0.05$) were detected, mean value differences were analysed using Tukey Test.

Results and Discussion

Food Intake and Body Weight Gain Body weight and food intake data are shown in Table 2. Estimated daily intake *per* rat was similar for all the groups. Weight gain did not show significant differences except in the case of ***CAI*** group, which presented a greater value ($p < 0.05$). In addition, ***CAI*** group presented a food efficiency % (FE %) twice the other groups. Since the only difference between groups was the diet composition, a possible explanation for the increased FE % value in ***CAI*** group could be related with an improved amino acid profile, due to the mixture of casein and amaranth proteins (10.7 % of casein protein was replaced by amaranth protein). Amino acid score values corrected by protein digestibility (PDCAAS) values calculated for protein mixture in ***CAI*** diet (0.84) were higher than those for casein in ***C*** diet (0.72), indicating a greater protein quality of the first diet (Table 2). However, it is remarkable that for ***CholAI*** group (similar PDCAAS value than ***CAI***), no increment in the FE % value was registered (Table 2). We can hypothesize that the cholesterol intake would induce metabolic changes in the animals, which would not allow the total protein utilization. However, we do not have enough information to explain these facts. In this sense, it is important to mention that multiple mechanisms for feedback control of cholesterol synthesis converge on the rate-limiting enzyme in the pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase. This complex feedback regulatory system is mediated by sterol and nonsterol metabolites of mevalonate [17].

Table 2 Body weight and food intake of rats

Group	Weight gain (g) [1]	Food intake (g/rat x day)	Food Efficiency (%) [2]	PDCAAS[3]
C	27 ± 16[a]	16.4	0.79	0.72
CAI	65 ± 19[b]	15.2	2.04	0.84
CE	18 ± 5[a]	17.1	0.50	0.72
Chol	1 ± 33[a]	16.1	0.03	0.72
CholE	26 ± 8[a]	15.3	0.81	0.72
CholAI	29 ± 13[a]	15.6	0.89	0.84

[1] Values are expressed as the mean ± SD ($n = 6$). Different letters indicate significant differences between groups ($\alpha = 0.05$)

[2] Food efficiency % = (weight gain/food intake) × 100

[3] Amino acid score values corrected by protein digestibility

Blood Pressure As it was previously mentioned, blood pressure was measured by an indirect method. In agreement with previous results [18], pressure obtained during the cuff inflation was higher than during the deflation. This difference is related to the local production of nitric oxide when the artery is compressed and the pressure during the inflation correlates better with the intravascular pressure [18]. In this way, values during the inflation corresponding to systolic pressure were analyzed. Measures did not show significant differences ($p > 0.05$) between groups at the beginning of the experiment, with a mean value of 142 ± 3 mmHg. Additional measures were performed after two and four weeks of specific feeding for each group. Animals from ***C***, ***CE***, ***Chol*** and ***CholE*** groups did not present significant differences during the experimental period ($p > 0.05$). Nevertheless, animals from ***CAI*** and ***CholA*** groups showed a significant ($p < 0.05$) diminution (25 and 27 mmHg in average, respectively) in the pressure values after feed diets containing amaranth protein isolate during four weeks (Fig. 1). Fritz et al. [2] have demonstrated an

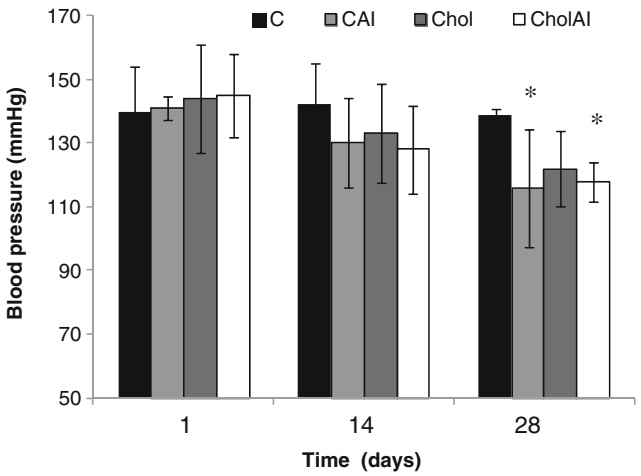

Fig. 1 Blood pressure at the beginning and after two and four weeks of specific feeding. Results are expressed as the mean ± SD ($n = 6$), * indicates a significant difference ($p < 0.05$) with respect to the initial (day 1) value

in vivo hypotensive activity of amaranth (*A. manteggazianus*) alcalase-hydrolysates dispensed by an intragastric way to spontaneously hypertensive rats (SHR), which could be attributed to a vasodilator effect of amaranth hydrolysates. In addition, an inhibitory effect of amaranth peptides on the angiotensin converting enzyme could exist, as it has been demonstrated in *in vitro* assays [1]. In the present work, some compounds generated by gastrointestinal digestion of amaranth protein isolate –maybe some peptides- have been able to enter the organism and cause some effects producing a reduction in blood pressure. High cholesterol content in the diet did not influence the hypotensive effect of the amaranth protein isolate.

Lipid Profiles

Plasma The addition of 1 % (*w*/*w*) chol to the diet did not produce significant changes ($p > 0.05$) neither in the plasma total cholesterol nor in the c-LDL and c-HDL, as it is evidenced by comparing values for ***C*** and ***Chol*** groups (Table 3). These results contradict others in the literature, in which the addition of 1 % (*w*/*w*) chol in diets of Wistar rats produced a plasma chol increment of 23 % in 28 days [3] or 62 % in 32 days [19]. Increment in the c-LDL and diminution in the c-HDL was also observed [19]. The presence of E in the diets (groups ***CE*** and ***CholE***) did not have any effect on plasma total, LDL and HDL cholesterol levels with respect to their corresponding control groups (Table 3). The addition of 2.5 % (*w*/*w*) AI in diet containing chol (group ***CholA***) produced a significant reduction ($p < 0.05$) of total cholesterol but no significant changes in the c-LDL and c-HDL levels. Other authors have proven a similar effect in hamsters using 12.5 % (*w*/*w*) amaranth (*A. cruentus*) protein isolates in diets added with 0.05 % (*w*/*w*) chol, observing a reduction of about 27 % in plasma total cholesterol [4]. In our case, reduction was about 16 % (2.5 % amaranth, 1 % chol). In another way, animals from ***CAI*** group (2.5 % (*w*/*w*) amaranth, without chol) presented a significant increment ($p < 0.05$) in plasma total cholesterol and c-LDL (Table 3). C-HDL/total cholesterol and c-LDL/total chol ratios were statistically similar ($p > 0.05$) for all the groups with values between 43 and 53 % and 38–50 %, respectively. According to the present results, c-VLDL represented only a very small proportion (0.02–0.04 %) of the total plasma cholesterol. Lipid profile of rats is different from the human. Rats have a very efficient mechanism for the removal of remaining chylomicrons and VLDL from blood circulation, producing low levels of c-LDL. In addition, they present a greater accumulation of c-HDL due to the absence of the cholesterol ester transfer protein (CETP), the main responsible in humans for the transference of the cholesterol esters of HDL to lower density lipoproteins [20]. Plasma triglycerides did not show significant differences ($p > 0.05$) between ***C***, ***CE*** and ***CAI*** groups. However, they presented a significant diminution ($p < 0.05$) due to the addition of 1 % (*w*/*w*) chol in the diet, with no differences due to the presence of E or AI (***Chol***, ***CholE*** and ***CholAI*** groups, respectively) (Table 3). Chen et al. [13] also found a significant diminution in the triglycerides values because of the addition of 1 % chol in the diet of mice.

Table 3 Plasma and liver lipid profiles for each experimental group

PLASMA

Group	Total cholesterol (g/L)	HDL-c (g/L)	LDL-c (g/L)	LDL-c/HDL-c (g/L)	Triglycerides (g/L)
C	$0.50 \pm 0.05^{a,b}$	$0.27 \pm 0.03^{a,b}$	$0.19 \pm 0.02^{a,b}$	0.73 ± 0.07^{a}	$0.21 \pm 0{,}04^{a}$
CE	0.55 ± 0.04^{a}	$0.24 \pm 0.01^{a,c}$	$0.28 \pm 0.04^{a,c}$	1.2 ± 0.2^{a}	$0.15 \pm 0{,}03^{a,b}$
CAI	0.71 ± 0.07^{d}	0.33 ± 0.09^{b}	0.3 ± 0.1^{c}	1.2 ± 0.6^{a}	$0.18 \pm 0{,}06^{a,c}$
Chol	$0.49 \pm 0.03^{a,b}$	$0.23 \pm 0.03^{a,c}$	$0.24 \pm 0.05^{a,b}$	1.1 ± 0.4^{a}	$0.13 \pm 0{,}04^{b,c}$
CholE	$0.44 \pm 0.03^{b,c}$	$0.23 \pm 0.02^{a,c}$	0.19 ± 0.02^{b}	0.8 ± 0.1^{a}	$0.12 \pm 0{,}04^{b,c}$
CholAI	0.41 ± 0.02^{c}	0.19 ± 0.02^{c}	$0.20 \pm 0.03^{a,b}$	1.0 ± 0.3^{a}	$0.10 \pm 0{,}03^{b}$

LIVER

Group	Total lipids (mg/g)	Total cholesterol (mg/g)	Triglycerides (mg/g)	Triglycerides %*	Cholesterol %*
C	44 ± 12^{a}	1.5 ± 0.2^{a}	8 ± 2^{a}	18 ± 7^{a}	4 ± 1^{a}
CAI	22 ± 8^{b}	2.1 ± 0.1^{a}	10 ± 3^{a}	46 ± 9^{b}	10 ± 2^{b}
Chol	81 ± 15^{c}	3.8 ± 0.5^{b}	17 ± 5^{c}	22 ± 5^{a}	5 ± 1^{a}
CholAI	55 ± 9^{a}	3.1 ± 0.5^{c}	9 ± 1^{a}	18 ± 2^{a}	6 ± 1^{a}

C (basal diet), Chol (chol 1 %, *w*/*w*), CE (α-tocopherol (E) 0.005 %, *w*/*w*), CholE (chol 1 % (*w*/*w*) + E 0.005 %, *w*/*w*), CAI (amaranth protein isolate AI 2.5 %, *w*/*w*), CholAI (chol 1 % (*w*/*w*) + AI 2.5 %, *w*/*w*). Values are expressed as the mean value ± SD ($n = 6$). Different letters in the same column indicate significant differences between groups ($\alpha = 0.05$). * Ratios respect to total lipids

Liver As can be seen in Table 3, cholesterol and triglycerides represented a minor proportion of the total lipids in all cases. Christie [21] reported that more than 80 % of the total lipids in the liver corresponded to phospholipids, being the phosphatidylcholine the major one. Similar results were recorded in livers of Wistar rats by Itou and Akahane [22]. Table 3 shows greater significant ($p < 0.05$) values of total lipids, cholesterol and triglycerides for ***Chol*** group compared with ***C*** group. According to this, previous works have demonstrated cholesterol and triglycerides increments in liver due to the addition of cholesterol in diets [13, 22]. Hypercaloric or lipid-rich diets induced the deposition of triglycerides in liver (steatosis) through different mechanisms [23], while an increment of 90 % of esterified cholesterol in liver of rats fed with diets containing 1 % *w/w* chol has been reported [3]. In the present work, 1 % (*w/w*) chol (***Chol***) during four weeks did not produce an increment in the plasma cholesterol but induced an increment in the liver lipids (total lipids, triglycerides and cholesterol) in Wistar rats, suggesting that dietary cholesterol would have first an effect on the liver lipids and probably it would later produce hypercholesterolemia. It has been demonstrated that Wistar rats are remarkably resistant to hypercholesterolemia. After intake of 1 % (*w/w*) chol, its synthesis was substantially reduced but after accumulation of hepatic cholesterol, the hepatic LDL receptor was upregulated with a substantial increase in its expression. Thus, the excess cholesterol is efficiently cleared from the plasma, being the LDL receptor implicated in this process [24]. The addition of 2.5 % (*w/w*) AI to high-cholesterol diet (***CholAI***) produced a significant reduction ($p < 0.05$) in liver total lipids, cholesterol and triglycerides compared with the control group ***Chol*** (Table 3), suggesting a suppressive effect on the liver deposition of lipids. Escudero et al. [3] reported a similar behaviour in Wistar rats feeding a diet containing 1 % (*w/w*) chol, but with a total replacement of casein by amaranth proteins. This is an almost 10-fold higher proportion (23 %, *w/w*) of amaranth (*A. cruentus*) protein concentrate, which contained about 52 % proteins, 6 % lipids (containing squalene) and a high proportion of soluble and insoluble dietary fiber (about 34 %). It is important to highlight that the effect obtained by these authors [3] was greater than in the present work probably due to a higher amount of amaranth as well as the presence of other possible active components (fiber, squalene, etc) in their protein concentrates. They proposed an inhibition of the cholesterol esterification because of some amaranth component, producing the reduction in the liver cholesterol. In addition, a reduction in the expression and activity of the fatty acid synthase (FAS) was recorded, with the consequent diminution in the fatty acids and triglycerides synthesis in the liver. Similar mechanisms could be acting in the present experiments. Animals feeding AI in absence of dietary chol presented a different behavior. Group ***AI*** showed a significant diminution ($p < 0.05$) in the total liver lipids in comparison with ***C*** group, but no differences in the cholesterol and triglycerides contents (Table 3), suggesting that total lipids diminution is related to other lipid fractions (likely phospholipids). A possible cause that could, at least partially, explain these results is the fact that the amaranth seeds contain squalene and it could partially remain in the protein isolates [4]. The squalene is an intermediary in the cholesterol synthesis route and it is an inhibitor of the HMG-coA reductase [25]. In this way, the intake of squalene could produce an inhibition of the tissue cholesterol biosynthesis. In the case of the ***CAI*** groups, a kind of "deficit" of cholesterol could result, inducing an increment in the mobilization of the previously stored cholesterol and, thus, increasing the plasma levels. In the case of ***CholAI*** group, the dietary cholesterol would avoid the "deficit", allowing the observation of the hypocholesterolemic effect of AI.

Faeces Because one of the main mechanisms to reduce plasma cholesterol is the increment in its faecal excretion, cholesterol contents in faeces of animals from ***Chol*** and ***CholAI*** were analyzed. The amount of daily faeces did not present significant ($p > 0.05$) differences between both groups (Fig. 2). However, faecal cholesterol content was significantly lower ($p < 0.05$) for animals from group ***Chol*** compared to group ***CholAI*** (Fig. 2), suggesting an increment in the excretion of cholesterol due to the intake of 2.5 % (*w/w*) AI. Escudero et al. [3] evidenced an increment in the excretion of cholesterol in rats feeding amaranth protein concentrate (23 %, *w/w*) along 28 days and they attributed this effect to the presence of soluble fiber and saponins. However, evidence for the potential action of peptides exists in the literature and they can exert their functions via bile acid-binding and disruption of cholesterol micelles in the gastrointestinal tract. Their activity depends on their physicochemical properties including hydrophobicity of amino acid residues, although these structure–function relationships are not totally understood [26]. Previous *in vitro* assays in our lab [unpublished work] have demonstrated a reduction of the micellar solubility of

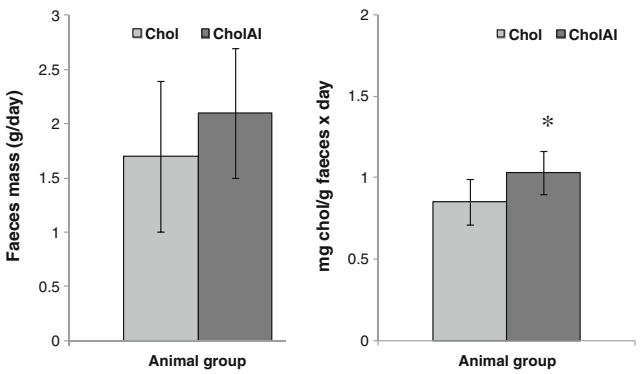

Fig. 2 Faeces mass and cholesterol in faeces collected during the last three days of the experiment for ***Chol*** and ***CholAI*** groups. Results are expressed as the mean ± SD ($n = 6$), *indicates significant differences ($p < 0.05$) between groups

cholesterol due to the presence of an amaranth (*A. hypocondriacus*) protein hydrolysate, suggesting that some peptides could compete with cholesterol molecules for a site on the micelles, reducing the cholesterol solubility and, consequently, its absorption. Mendonça et al. [4] demonstrated an increased excretion of cholesterol in hamsters feeding a diet containing 0.5 % (*w/w*) cholesterol and 22.5 % (*w/w*) amaranth (*A. cruentus*) protein isolate along three weeks, attributing the effect to the presence of peptides generated by gastrointestinal digestion.

Antioxidant Status

Plasma All the groups showed similar FRAP values except ***CholAI***, which presented a significantly higher FRAP value ($p < 0.05$) (Table 4). This indicated a greater reducing capacity of these plasmas, that is, a higher concentration of antioxidant molecules. It is important to note that diets added with E (***CE*** and ***CholE***) did not show any increment in the FRAP values and this may be possibly related to its liposoluble character. AI was able to increase the FRAP value in the diet containing cholesterol (***CholAI***) but not in the diet without cholesterol (***CAI***). TBA values demonstrated that the addition of 1 % (*w/w*) chol to the diet (***Chol***) did not produce significant changes ($p > 0.05$) in lipid oxidation products compared to ***C*** group (Table 4). Cholesterol-rich diets cause an increment in the lipid peroxidation since the hypercholesterolemia induced an increment in the cholesterol content of blood and endothelial cells, activating these cells and producing an increment in the production of reactive oxygen species (ROS) [27, 28]. This effect was not registered in the present work and this could be related to the fact that plasma cholesterol was not increased in the present conditions either. The addition of E in the absence (***CE***) and the presence (***CholE***) of cholesterol produced a significant diminution in the TBA values ($p < 0.05$), evidencing the known antioxidant capacity of this compound, which is one of the main antioxidants at the membrane level. The addition of AI in the diet without cholesterol (***CAI***) had no effect on the production of secondary lipid oxidation products, but it produced a significant reduction ($p < 0.05$) in the TBA value when chol (1 %) was present in the diet (***CholA***), with the last group achieving similar values as ***CholE*** group (Table 4). SOD catalyzes the dismutation of superoxide anion to oxygen and hydrogen peroxide, being an important antioxidant cell defence, which presents intracellular and extracellular (EC-SOD) isoenzymes. SOD activity % of plasmas can be observed in Table 4. There were not significant differences ($p > 0.05$) among the different groups. However, we would like to remark that both group ***CholE*** and group ***CholAI*** showed a tendency to lower SOD activity values than ***Chol*** (significant with $\alpha = 0.1$), suggesting a tendency to diminution when E or AI are in the diets containing also chol.

Liver There were not significant differences ($p > 0.05$) between ***C*** and ***Chol*** groups for the TBA assay (Table 4), indicating again no effect of the addition in the diet of 1 % (*w/w*) chol on the formation of lipid oxidation products. Besides, addition in the diet of E or AI without chol did not have any significant effect ($p > 0.05$) on the TBA number of the livers. In another way, in the presence of dietary chol, both E (***CholE***) and AI (***CholA***) produced a significant diminution ($p > 0.05$) in the TBA numbers in comparison to the control group ***Chol***, evidencing –as in the case of plasma determinations- a protector effect of these substances against the oxidative stress. In Table 4 the SOD activity % expressed *per* mg of liver is shown. Groups ***C*** and ***Chol*** did not show significant differences ($p > 0.05$). SOD level is increased as an adaptative response to the oxidative stress [29]. In this case, this response could not be evidenced as the chol added in the diet did not modify the oxidation level in the experimental time. When E or AI were added in the diets without cholesterol (***CE*** and ***CAI***, respectively), there were not significant changes ($p < 0.05$) in the SOD activity (Table 4). ***CholE*** diet produced

Table 4 Antioxidant status of plasma and liver for each experimental group

Group	PLASMA			LIVER	
	FRAP value (mmoles Fe^{+2}/L)	TBA value (mmoles MDA/mL)	SOD activity %	TBA value (mmol MDA/mg liver)	SOD activity (%/100 μg liver)
C	0.21 ± 0.05^{a}	4.8 ± 0.8^{a}	69 ± 8^{a}	0.22 ± 0.05^{a}	$19.5 \pm 0.5^{a,b}$
CE	0.21 ± 0.05^{a}	1 ± 1^{b}	61 ± 14^{a}	0.20 ± 0.01^{a}	20.8 ± 0.5^{a}
CAI	0.28 ± 0.07^{a}	5 ± 1^{a}	65 ± 7^{a}	$0.16 \pm 0.05^{a,b}$	20.3 ± 0.7^{a}
Chol	0.28 ± 0.06^{a}	5.0 ± 0.6^{a}	75 ± 7^{a}	0.21 ± 0.02^{a}	20.7 ± 0.9^{a}
CholE	0.18 ± 0.04^{a}	1.1 ± 0.2^{b}	60 ± 9^{a}	$0.17 \pm 0.04^{a,b}$	19 ± 1^{b}
CholAI	0.4 ± 0.1^{b}	1.5 ± 0.4^{b}	60 ± 3^{a}	0.13 ± 0.03^{b}	20.2 ± 0.4^{a}

C (basal diet), Chol (chol 1 %, *w/w*), CE (α-tocopherol (E) 0.005 %, *w/w*), CholE (chol 1 % (*w/w*) + E 0.005 %, *w/w*), CAI (amaranth protein isolate AI 2.5 %, *w/w*), CholAI (chol 1 % (*w/w*) + AI 2.5 %, *w/w*). Values are expressed as the mean value ± SD ($n = 6$). Different letters in the same column indicate significant differences between groups ($\alpha = 0.05$)

a significant diminution ($p < 0.05$) of the SOD activity in liver; while ***CholAI*** diet did not show significant differences compared with ***Chol*** group, indicating that AI had no effect on the SOD activity in liver in the present conditions. In agreement with the lack of cholesterol increment in the plasma, ***Chol*** group did not register an induction of the oxidative stress – measured by FRAP, TBA assay and SOD activity- compared with ***C*** group. Although lipids accumulation was registered in livers of animals from ***Chol*** group, this tissue neither presented increments in TBA numbers nor changes in the SOD activity. The exact mechanisms of liver inflammation and oxidative stress by dietary cholesterol are not yet well established. In Sprague-Dawley rats, cholesterol feeding (1 % *w*/*w*, 12 weeks) markedly elevated lipid peroxidation in the livers [30]. Authors proposed that since elevated liver cholesterol content is associated with an increased rate of liver cholesterol degradation and the lipid peroxidation is a normal process in cholesterol catabolism, the greater rate of cholesterol degradation may increase the rate of hepatic lipid peroxidation. On the other hand, cholesterol feeding also increased hepatic fatty acid or triglycerides levels, as well as the concentration of polyunsaturated fatty acid due to the formation of cholesterol ester with these fatty acids. This fact would aggravate the susceptibility of the tissues to lipid peroxidation. In addition, cholesterol feeding also resulted in a marked reduction in the activity of glutathione peroxidase in the liver. In another study, albino rats fed with a diet containing 2 % *w*/*w* cholesterol during three months showed significant increments in the malondialdehyde, hydroperoxides and conjugated dienes, as well as a reduction in SOD and catalase activities of livers and other tissues [31]. In our case, the intake time was probably not enough to produce detectable changes in the lipid oxidation products or SOD activity. Additionally, ***CE*** group did not show any antioxidative effects on liver, as could be expected. The addition of AI (2.5 %, *w*/*w*) to the diet without cholesterol (***CAI***) did not produce any change in the antioxidant status of plasma or liver. However, the presence of chol + AI (***CholA***) induced an increment in the antioxidant capacity of the plasma and a reduction in the secondary lipid oxidation products level of plasma and liver. Diverse hypotheses can be postulated in order to explain these results. One of them could be that the increment in the reducing capacity of plasmas in ***CholAI*** group was not related to a direct action of amaranth components present in plasma –which at first should be also in the plasma of animals from ***CAI*** group- but to some indirect mechanism induced by amaranth components. Previous studies in our lab have demonstrated that, after *in vitro* gastrointestinal digestion, amaranth proteins are able to generate antioxidant peptides [7, 8]. However, antioxidant peptides should be absorbed and resist the action of peptidases in order to be active in the different locations of the organism. In this way, it is possible that in the present conditions, the amount of active peptides in the organisms were not enough in ***CAI*** group. But, considering that antioxidant effects were registered in ***CholAI*** group, a differential absorption of peptides due to the presence of cholesterol could be postulated. In this sense, it is important to mention that the hydrophobic properties of peptides are important to their antioxidant activity. Wistar rats fed with "douchi" (a soy fermented food) presented an increased SOD activity and a decreased TBA value in liver, with diminished lipidosis, mitochondrial deterioration and nuclear rupture of the hepatocytes. These protective effects have been attributed to a synergism between the antioxidant peptides and the tocopherols [31]. Nazeer et al. [32] demonstrated that Wistar rats fed with a diet containing an antioxidative peptide from fish muscle were protected against the ethanol induced oxidative damage. The involved peptide presented hydrophobic characteristics and could be successfully absorbed by the animals.

Conclusions

The addition of a minor proportion of AI (2.5 %, *w*/*w*, about 10 % amaranth protein with respect to the total protein) to the diet (1 % *w*/*w* chol) of Wistar rats produced a reduction in the plasma and liver cholesterol after 28 days, with an increment in the faecal cholesterol excretion as one of the possible mechanisms of action. In addition, the intake of AI in the presence of dietary chol resulted in an increment in the reducing capacity, a diminution in the secondary oxidation products and in the SOD activity of plasma, as well as a reduction in secondary products in liver. Also, the intake of AI in the presence or absence of dietary chol produced a reduction in blood pressure after 28 days. In another way, the diet containing AI in the absence of chol induced a higher body weight increment as well as increased total cholesterol content in plasma and changes in the liver lipids, suggesting some metabolic effect of amaranth, which could not be completely explained with the present results. These results constitute a first approach to the *in vivo A. manteggazianus* protein isolate activity, suggesting it as a potential functional ingredient with hypotensive, hypolipidemic and antioxidant activities and confirming *in vitro* studies which have included simulation gastrointestinal digestion of AI and the identification of the peptides present in the digests. Further experiments should be done in order to obtain the necessary information to explain the mechanisms of action involved in these activities.

Acknowledgments This work has been supported by an Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Argentina) project (PICT 2007-1119) and a CONICET project (PIP 2008-01330). Authors Rinaldi, Añón and Tironi are members of CONICET (Argentina). Authors want to thank to Nilda Reinaudi (Facultad de Agronomía, UNLPam, Argentina) and Guillermo Peiretti (Facultad de Agronomía, UNRC, Argentina) for the provision of amaranth seeds, and to Cátedra de Nutrición y Bromatología (Facultad de Farmacia y Bioquímica, UBA,

Argentina) for the collaboration in the diets preparation.

Compliance with Ethical Standards

Conflict of Interest M. B. Lado declares that she has no conflict of interest.

J. Burini declares that she has no conflict of interest.

G. Rinaldi declares that she has no conflict of interest.

M. C. Añón declares that she has no conflict of interest.

V. Tironi declares that she has no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

1. Vecchi B, Añón M (2009) ACE inhibitory tetrapeptides from *Amaranthus hypochondriacus* 11S globulin. Phytochem. 70(7): 864–870
2. Fritz M, Vecchi B, Rinaldi G, Añón M (2011) Amaranth seed protein hydrolysates have *in vivo* and *in vitro* antihypertensive activity. Food Chem 126:878–884
3. Escudero N, Zirulnik F, Gomez N, Mucciarelli S, Giménez M (2006) Influence of a protein concentrate from *Amaranthus cruentus* seeds on lipid metabolism. Exp Biol Med 231:50–59
4. Mendonça S, Saldiva P, Cruz R, Arêas A (2009) Amaranth protein presents cholesterol-lowering effect. Food Chem 116:738–742
5. Nsimba R, Kikuzaki H, Konishi Y (2008) Antioxidant activity of various extracts and fractions of *Chenopodium quinoa* and *Amaranthus* spp. seeds. Food Chem 106:760–766
6. López V, Razzeto G, Giménez M, Escudero N (2011) Antioxidant properties of *A. hypochondriacus* seeds and their effect on the liver of alcohol-treated rats. Plant Foods Hum Nutr 66:157–162
7. Orsini Delgado M, Tironi V, Añón M (2011) Antioxidant activity of amaranth protein or their hydrolysates under simulated gastrointestinal digestion. LWT-Food Sci Technol 44:1752–1760
8. Orsini Delgado M, Galleano M, Añón M, Tironi V (2015) Amaranth peptides from gastrointestinal digestion: antioxidant activity against physiological reactive species. Plant Foods Human Nutr 70:27–34
9. Martinez N, Añón MC (1996) Composition and structural characterization of amaranth protein isolates. An electrophoretic and calorimetric study. J Agric Food Chem 44:2523–2530
10. Reeves P, Nielsen F, Fahey Jr G (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition *ad hoc* writing committee on the reformulation of the AIN-76A rodent diet. Diet J Nutr 123:1939–1951
11. Friedewald W, Levy R, Fredrickson D (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 18(6): 499–502
12. Folch J, Lees M, Sloane-Stanley G (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
13. Chen G, Luo Y, Ji B, Li B, Su W, Xiao Z, Zhang G (2011) Hypocolesterolemic effects of *Auricularia auricula* ethanol extract in ICR mice fed a cholesterol-enriched diet. J Food Sci Technol 48(6):692–698
14. Bragagnolo N, Rodríguez-Amaya D (2003) Comparison of the cholesterol content of Brazilian chicken and quail eggs. J Food Composition Anal 16:147–153
15. Benzie I, Strain J (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239:70–76
16. Yagi K (1976) A simple fluorometric assay for lipoperoxides in blood plasma. Biochem Med 15:121–216
17. Friesen J, Rodwell, V. (2004). The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. Genome Biology, 5, 11, Article 248.
18. Fritz M, Rinaldi G (2007) Influence of nitric oxide-mediated vasodilation on the blood pressure measured with the tail-cuff method in the rat. J Biomed Sci 14(6):757–765
19. Czerwinski J, Bartnikowska E, Leontowicz H, Langeb E, Leontowicz M, Katrich E, Trakhtenbergd S, Gorinstein S (2004) Oat (*Avena sativa* L.) and amaranth (*Amaranthus hypochondriacus*) meals positively affect plasma lipid profile in rats fed cholesterol containing diets. J Nutr Biochem 15:622–629
20. Oschry Y, Eisenberg S (1982) Rat plasma lipoproteins: re-evaluation of a lipoprotein system devoid of cholestryl ester transfer activity. J Lipid Res 23:1099–1106
21. Christie W (1985) Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. J Lipid Res 26:507–512
22. Itou K, Akahane Y (2010) Effect of extracts from narezushi, a fermented mackerel product, on cholesterol metabolism in wistar rats. J Food Sci Technol 76:537–546
23. Frota K, Mendonça S, Saldiva R, Cruz R, Arêas J (2008) Cholesterol-lowering properties of whole cowpea seed and its protein isolate in hamsters. J. Food Sci 73(9):235–240
24. Roach P, Balasubramaniam S, Hirata F, Abbey M, Szanto A, Simons L, Nestel P (1993) The low-density lipoprotein receptor and cholesterol synthesis are affected differently by dietary cholesterol in the rat. Biochim Biophys Acta 1170:165–172
25. Qureshi A, Lehmann J, Peterson D (1996) Amaranth and its oil inhibit cholesterol biosynthesis in 6-week-old female chickens. J Nutr 126:1972–1978
26. Howard A, Udenigwe C (2013) Mechanisms and prospects of food protein hydrolysates and peptide-induced hypolidaemia. Food Funct 4:40–51
27. Kok F, van Poppel G, Melse J, Verheul E, Schouten E, Kruyssen D, Hofman A (1991) Do antioxidants and polyunsaturated fatty acids have a combined association with coronary atherosclerosis? Atherosclerosis 86(1):85–90
28. Prasad K, Kalra J (1989) Experimental atherosclerosis and oxygen free radicals. Angiology 40:835–843
29. Gupta V, Lahiri S, Sultana S, Tulsawani T, Kumar R (2010) Antioxidative effect of *Rhodiola imbricata* root extract in rats during cold, hypoxia and restraint (C–H–R) exposure and post-stress recovery. Food Chem Toxicol 48:1019–1025
30. Tsai C (1975) Lipid peroxidation and glutathione peroxidase activity in the liver of cholesterol-fed rats. J Nutr 105:946–951
31. Wang D, Wang L, Zhu F, Zhu J, Chen X, Zou L, Saito M, Li L (2008) *In vitro* and *in vivo* studies on the antioxidant activities of the aqueous extracts of douchi (a traditional Chinese salt-fermented soybean food). Food Chem 107:1421–1428
32. Nazeer R, Kumar N, Ganesh R (2012) *In vitro* and *in vivo* studies on the antioxidant activity of fish peptide isolated from the croaker (*Otolithes ruber*) muscle protein hydrolysate. Peptides 35:261–268