Cholesterol-Lowering Properties of Amaranth Grain and Oil in Hamsters[1]

Alvin Berger[a], Gérard Gremaud[,2], Marcel Baumgartner[b], Dietrich Rein[b,5], Irina Monnard[b], Elena Kratky[b], Wolfgang Geiger[b,3], Joseph Burri[c], Fabiola Dionisi[b], Martin Allan[b,4] and Pierre Lambelet[b,*]

[a] Manager Lipidomics™, Cytochroma Inc., 330 Cochraine Drive, Markham, ON L3R 8E4, Canada
[b] Nestlé Research Centre, 1000 Lausanne 26, Switzerland
[c] Nestlé PTC Orbe, 1350 Orbe, Switzerland

[1] The hamster work appeared in part in FASEB J. 14: A250, 2000
[2] Current affiliation: Swiss Federal Office of Public Health, 3000 Bern, Switzerland
[3] Current affiliation: Bayern Innovativ GmbH, 90403 Nürnberg, Germany
[4] Current affiliation: Biozentrum der Universität Basel, 4056 Basel, Switzerland
[5] Current affiliation: BASF Plant Science Holding, 67114 Limburgerhof, Germany
*To whom correspondence should be addressed at Nestlé Research Centre, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

Received for publication: May 21, 2002

Abstract: Amaranth was an important ancient grain and has current nutritional potential, being high in protein, fiber, lysine, magnesium, calcium, and squalene. Limited, inconsistent evidence demonstrates amaranth grain or oil can lower cholesterol in animal models. In the present study, hamsters received hypercholesterolemic diets consisting of a control, 10 or 20% *Amaranthus cruentus* grain, or 2.5 or 5% crude amaranth oil for four weeks. Amaranth oil (5%) decreased total and non-high-density lipoprotein (HDL) cholesterol by 15 and 22%, respectively, compared to control. Amaranth grain (20%; providing 1.4% amaranth oil) lowered non-HDL cholesterol and raised HDL cholesterol. Amaranth grain and oil decreased very low-density lipoprotein (VLDL) cholesterol by 21–50%; and increased fecal excretion of particular neutral sterols and the bile acid ursodeoxycholate. Amaranth oil (5%) additionally increased the cholesterol synthesis rate, possibly due to compensatory mechanisms; and decreased hepatic cholesterol ester, indicating reduced cholesterol ester availability for VLDL secretion and consistency with reduced VLDL cholesterol. Amaranth thus affected absorption of cholesterol and bile acids, cholesterol lipoprotein distribution, hepatic cholesterol content, and cholesterol biosynthesis. Amaranth grain and oil did not affect these pathways identically.

Key words: Absorption, amaranth, bile acid, cholesterol, hamster, synthesis

Abbreviations used: ACAT, acyl-CoA: cholesterol acyltransferase; ANOVA, analysis of variance; DPBS, Dulbecco's phosphate buffered saline; FSR, fractional synthesis rate; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; IRMS, isotope ratio mass spectroscopy; LDL, low-density lipoprotein; TAG, triacylglycerol; VLDL, very low-density lipoprotein.

Introduction

Amaranth was a staple crop of the Aztecs and was also cultivated by the Mayas, Incas, and other pre-Columbian peoples [1]. Although several amaranth species are considered weeds, amaranth is a productive, hardy crop, with food potential for both Third World and industrialized countries [1]. Amaranth grain is rich in protein, fiber, magnesium, calcium, squalene, protein, and lysine, the latter being limited in many grains [1]. Amaranth has a healthy fatty acid profile similar to corn [2]. In addition to its promising nutritional qualities, amaranth may also be valuable for diabetics [3] and coeliac patients [4].

Amaranth's cholesterol-lowering properties have been investigated in animal models and humans, using differently processed amaranth of various species and cultivars, with conflicting results [5–13].

In this study, we describe the dietary effects of milled, extruded amaranth grain and crude amaranth oil using the established hypercholesterolemic hamster model, rather than rodent models. We evaluated effects of amaranth on cholesterol lipoprotein distribution, plasma triacylglycerol (TAG) concentrations, liver cholesterol content, neutral and fecal bile acids, and cholesterol biosynthesis to better understand the mechanism of action and possible active components in amaranth. Our results advance the understanding of how amaranth components affect cholesterol metabolism, and provide insight into which amaranth components could be enriched to produce a future amaranth plant with improved cholesterol-lowering properties.

Materials and Methods

Experimental animals. Seventy-five adult male golden Syrian hamsters (Hsdt Han: AURA), 80–100 g initially, were obtained from Harlan UK Ltd (Blackthorn, England). Hamsters were caged individually in wire cages with a daily 07:00–19:00 light period. Temperature was maintained at 23 ± 2°C and humidity at 55 ± 10%. For four weeks preceeding experimentation, hamsters received commercial chow (Nafag 924, Gossau, Switzerland); then during a three-day adaptation period, respective ratios of chow:control diets of 75:25, 50:50, and 25:75; and after random equilibration by body weight into five groups of 15, the experimental diets. Animal procedures were authorized by Service Vétérinaire du Canton de Vaud, Switzerland, permit 1175.

Diets. Diets contained 20% protein, 53% carbohydrate, 20% fat, 2% fiber, and 5% minerals, vitamins, methionine, and cholesterol (Table Ia). Hypercholesterolemic diets [14] contained 14% of coconut oil that was hydrogenated to near completion and 0.12% cholesterol. Amaranth oil was replaced by corn oil in the control diet due to its similar fatty acid profile [2, 15–17].

Amaranth flakes were prepared by milling *Amaranthus cruentus seeds* (Nu-World Amaranth, Naperville, IL) with a hammermill, and extruding with a BC45H twin-extruder (Clextral Inc., Firminy, France). During extrusion, 15% H_2O, 0.70% sunflower oil, 0.43% NaH_2PO_4, 0.34% NaCl, 0.14% deodorized paprika, and 0.04% Covi-ox® T30P antioxidant (Cognis Nutrition & Health, LaGrange, IL) were added. Moisture content was then reduced during extrusion and residence on a fluidized bed. Flakes were either re-crushed in a hammermill for preparation of amaranth grain diets; extracted with hexane for oil production; or mixed into food products for a human pilot study [13]. Amaranth oil was prepared by Soxhlet-extracting amaranth flakes three times with distilled technical grade hexane at 60°C without further refining. Amaranth seeds contained 7.0% oil. Paprika was added to both wheat flakes and amaranth flakes for the human pilot study to normalize the reddish color of amaranth.

Following mixing of diets, the 2.5 and 5% amaranth oil diets were dried in a vacuum oven at 40°C overnight to reduce volatiles and enhance odor and palatability. Diets were stored in freezer bags at –40°C under N_2 in weekly allotments; open containers were stored at 4°C. Dietary fatty acids were analyzed following lipid extraction, trans-

Table Ia: Variable Components of Diets (g component/100 g Diet, wet weight)

	Control	10% grain	20% grain	2.5% oil	5% oil
Amaranth flakes[a]	–[b]	10.0	20.0	–	–
Amaranth oil[a]	–	–	–	2.5	5.0
Corn oil[c]	5.0	4.3	3.6	2.5	–
Casein[d,e]	20.0	18.4	16.8	20.0	20.0
Corn starch[e]	38.0	30.7	23.4	38.0	38.0
Cellulose[e]	1.9	1.5	1.0	1.9	1.9

Diets contained 15% dextrose (Dyetrose 401476), 14% hydrogenated coconut oil (Dyet 400950), 3.5% mineral mix (Dyet 36001), 1.0% vitamin mix (Dyet 26001), 0.2% choline bitartrate (Dyet 400750), 0.3% DL-methionine (Dyet 402950; Dyets Inc., Bethlehem, PA), 1% fish oil (containing DL-α-tocopherol, ascorbyl palmitate, and lecithin; Hoffmann-La Roche, Basel, Switzerland), and 0.12% cholesterol (0.11-0-13 determined, Table Ic; C3292, Sigma, Buchs, Switzerland). Variable ingredients kept total protein (20%), fat (20% theoretical, 18.7–21.6% determined, Table Ib), carbohydrate (53%), and fiber (2%) constant. Minerals, vitamins, methionine, and cholesterol comprised 5%. Amaranth grain contained 7.14% fat; 10 and 20% amaranth grain diets provided 0.7 and 1.4% amaranth oil, respectively.
[a] See text for details; [b]– denotes 0.00%; [c]Obtained from SABO, Manno, Switzerland; [d]Vitamin-reduced; [e]Nestlé stock.

Table Ib: Fatty Acid Compositions of the Diets (g Fatty Acid/100 g Diet)

	Control	10% grain	20% grain	2.5% oil	5% oil
8:0	0.8	0.8	0.8	0.8	0.8
10:0	0.7	0.7	0.7	0.7	0.7
12:0	6.2	6.2	6.2	6.2	6.2
14:0	2.6	2.6	2.6	2.6	2.6
16:0	1.9	1.9	2.0	2.0	2.1
16:1n-7	0.1	0.1	0.1	0.1	0.1
18:0	1.8	1.8	1.8	1.9	1.9
18:1n-9	1.5	1.5	1.5	1.6	1.7
18:2n-6	2.7	2.6	2.4	2.2	1.8
20:5n-3	0.2	0.2	0.2	0.2	0.2
22:6n-3	0.1	0.1	0.1	0.1	0.1
Other identified and unidentified fatty acids[a]	0.1	0.1	0.1	0.1	0.1
Total fatty acids/100 g diet	18.8	18.8	18.7	18.6	18.5
Total g fat/100 g diet	20.3	18.7	20.0	21.6	19.9

[a] Including 15:0, 17:0, 20:0, 22:0, 24:0, 14:1n-5, 17:1n-7, 20:1n-9, 22:1n-9, 24:1n-9, 20:2n-6,18:3n-3, 18:3n-6, 20:3n-6,16:4n-3, 18:4n-3, 20:4n-3, 20:4n-6, 22:5n-3.

Table Ic: Sterol Content of the Diets (mg Sterols/100 g Diet)[a]

	Control	10% grain[b]	20% grain	2.5% oil	5% oil
Cholesterol[c]	115.5	106.3	105.9	119.6	133.2
α-Spinasterol[d]	–	26.4	23.5	35.8	44.0
Sitosterol	29.1	–	–	–	–
Campesterol	8.5	7.5	6.3	5.6	2.2
Stigmasterol	3.9	3.4	3.1	3.0	2.2
Δ^5-Avenasterol	1.9	1.7	1.3	2.2	2.7
Sitostanol	1.0	1.1	0.9	1.0	1.3
24-Methylene cholesterol	0.6	0.6	0.3	0.5	0.7
Δ^7-Ergosterol	0.0	1.1	1.4	11.1	24.9
Campestanol	0.4	0.3	0.3	0.4	0.6
Δ^7-Stigmasterol	0.3	1.1	1.3	11.2	23.5
Δ^7-Avenasterol	0.3	0.8	0.8	6.5	13.1
$\Delta^{5,24}$-Stigmastadienol	0.3	0.2	0.3	1.0	1.9
Unknown sterols	0.7	1.0	0.67	0.7	2.6

[a] Total fat was extracted from diets, quantified, and plant desmethyl sterols measured in total fat and re-expressed on a dietary wt% basis; [b] Unprocessed amaranth grain had the following wt% plant sterols: α-spinasterol 34.9; Δ^7-ergosterol 24.8; Δ^7-stigmasterol 22.2; Δ^7-avenasterol 12.9; Δ^5-avenasterol 1.6; $\Delta^{5,24}$-stigmastadienol 1.2; stigmasterol 1.0; campesterol 0.6; 24-methylene cholesterol 0.3; sitostanol 0.2; campestanol 0.04; and unknown sterols 0.3. There was 26.5 mg total sterols/g amaranth oil and 1.9 mg total sterols/g amaranth grain; [c] Cholesterol was added at 0.12g/100 g diet; [d] α-spinasterol and sitosterol may coelute.

esterification, and gas chromatographic analysis of fatty acid methyl esters (Table Ib). Dietary desmethyl sterols were determined following lipid extraction, transesterification, and liquid chromatography-gas chromatography (Table Ic). Nestlé in-house methodology was employed for fatty acid and sterol analyses.

Food intake and growth. Hamsters ate *ad libitum* amounts of diets and distilled water. Uneaten food was weighed and discarded daily to quantify food intake. Hamsters were weighed one month before the study. Hamsters that lost weight prior to study commencement were not included in the study. Hamsters were weighed once per week during experimentation.

Lipid blood parameters. Hamsters were randomly sacrificed with respect to treatment, day, and time on days 31–32, after a 16-hour fast, to obtain blood in the hamster's fasted state. Hamsters were terminated via isoflurane intoxication and exsanguination, and dorsal aortic blood was collected and spun at 2000 rpm, stored at –80°C, and analyzed for total cholesterol and TAG enzymatically (Kits 352 and 336, respectively; Sigma, Buchs, Switzerland). Orbital blood (500 µL) was removed from the eye with a sterile, heparinized glass capillary on day one and days 28–31 and analyzed to determine baseline total cholesterol (day one) and cholesterol synthesis rates (days 28–31).

Lipoprotein assays. Lipoproteins were separated with size-exclusion, high-performance liquid chromatography (HPLC). Plasma (10 µL) was diluted 1:1 (by volume) with Dulbecco's phosphate buffered saline (DPBS), pH 7.4, containing 0.02% sodium azide. Samples were injected onto a Superose 6HR fast protein liquid chromatography (FPLC) column (Pharmacia LKB Biotechnology, Piscataway, NJ), and lipoproteins eluted with DPBS at 0.6 mL/minute (S2100 solvent delivery pump; Sykam, Gilching, Germany). Lipoprotein cholesterol was determined with a post-column reactor, a mixing coil in a 37°C water bath, and an HPLC pump (Sykam S1100) to deliver cholesterol reagent (CHOD-PAP; Roche Diagnostics, Rotkreuz, Switzerland) at 0.1 mL/minute. Absorbance was recorded at 500 nm (Waters Absorbance Detector 484; Millipore, Millford, MA), and VLDL, LDL, and HDL cholesterol concentrations calculated from cholesterol fractions and total cholesterol.

Liver weights and cholesterol. Livers were excised, weighed, stored at –80°C, and cholesterol extracted as modified from Tercyak [18] as follows: liver sections (0.25 g) were mixed with H_2O to yield 1 mL volume, homogenized 30 seconds (Polytron homogenizer; Kinematica, Luzern, Switzerland), mixed with 1.33 mL MeOH, homogenized 30 seconds, and extracted with 2.66 mL $CHCl_3$; the aqueous layer was re-extracted with 2 mL $CHCl_3$/MeOH (88:12, by volume), organic layers evaporated under N_2, redissolved in 200 µL isopropanol, and total cholesterol determined in a 100 µL aliquot, as for

plasma. Free cholesterol was determined with a free cholesterol kit (274-47109 E; Wako, Neuss, Germany), and esterified cholesterol, by difference.

Cholesterol synthesis rates. On day 28, a bolus dose of D_2O (0.3% of body weight; D_2O 99.8%; Cambridge Isotope Laboratories, Andover, MA) was administered subcutaneously, followed by D_2O in the drinking water (D_2O/H_2O, 0.3:97.3, by weight) until experimental conclusion. For cholesterol fractional synthesis rate determination (FSR), orbital blood (200–300 µL) was analyzed 24–72 hours after the bolus dose. Cholesterol deuterium enrichment was determined on a Delta Plus XL Finnigan Thermoquest gas chromatography-pyrolysis-isotope ratio mass spectrometer (Bremen, Germany; IRMS); the D:H ratio was determined on a SIRA 12 IRMS system (VG Instruments, Crew, UK) [19]. FSR expressed in percent-change per day was calculated as the rate of incorporation of deuterium into free cholesterol over the 72-hour period [19].

Fecal bile acids and neutral sterols. Feces were quantitatively collected on days one, 14, and 29–31 through a double grid in the cages, freeze-dried overnight, and analyzed [20]. For bile acid analysis, 100 mg dried feces were spiked with 250 µL 0.8 mM nordeoxycholic acid internal standard (Steraloids, Newport, RI), and extracted three times with 4.5 mL of H_2O/EtOH (9:1, by volume). The solution was filtered, washed twice with 10 mL cyclohexane, and the residue treated with 2 mL 2,2-dimethoxypropane/1M HCl (7:1, by volume) to dissolve bile acid sulfates, and 5 mL 5M NaOH at 120°C for four hours to de-conjugate taurine and glycine. Following acidification with 4M HCl and extraction with two volumes of 3 mL diethyl ether, carboxy groups were methylated with 0.5 M methanolic HCl (Supelco, Buchs, Switzerland), and hydroxyl groups silylated with 1.5 mL Sylon HTP (pyridine/hexamethyldisilazane/trimethyl-chlorosilane, 9:3:1, by volume; Supelco, Buchs, Switzerland). Samples were separated by gas chromatography. For neutral sterol analysis, 100 mg freeze-dried feces were spiked with 250 µL 0.8 mM 5-α-cholestane internal standard, extracted with 1M NaOH in EtOH/H_2O (9:1, by volume), filtered, diluted with 6 mL H_2O, extracted with two volumes 10 mL cyclohexane, concentrated, derivatized with Sylon HTP, and sterols separated by gas chromatography.

Statistical analyses. All data were analyzed by analysis of variance (ANOVA) and Tukey's multiple comparison test, $p \leq 0.05$, unless indicated otherwise. The following variables were considered alone together as covariates in the ANOVA: total body weight change, total food intake, ratio of total body weight change: total food intake, and normalized food intake (average of eight ratios of food intake and weight, corresponding to the eight days for which the food intake data was available). Considering these covariables in the ANOVA did not affect the statistical conclusions reached regarding the various cholesterol blood parameters assessed. For simplicity, our results refer to analyses without covariate consideration.

Results

Food intake and growth. Hamsters fed the control diet gained more body weight (day 25 body weight – day one body weight) than those fed 2.5% amaranth oil (Table II). Food consumption (daily food intake summed from day 2 to day 26) was less for hamsters fed amaranth oil than those fed the control diet. Normalized food intake (daily food intake/weekly measured body weight, from day one to day 25) was statistically significantly less for hamsters fed 2.5% and 5% amaranth oil than those fed the control diet.

Lipid blood parameters. Baseline total cholesterol levels were 3.0–3.3mM. Changes in cholesterol were only statistically significant for the 5% oil group versus the control, although there was indication of decreased total cholesterol with increasing amaranth oil content. Relative to control, VLDL cholesterol was dramatically lowered with 20% grain and 2.5 and 5% oil; LDL cholesterol was not lowered by any of the treatments. The 10 and 20% grain groups had HDL cholesterol levels 22–25% higher than control groups.

The sum of VLDL + LDL (non-HDL cholesterol), was lowered 21–22% in the 20% grain and 5% oil groups. Non-HDL cholesterol was lowered by 18% in animals fed 2.5% oil, but the decrease was not statistically significant. The ratio of (VLDL + LDL)/HDL cholesterol was lower for

Table II: Food Intake and Growth after 4 weeks[1]

	Control	10% grain[b]	20% grain	2.5% oil	5% oil
BW change, d 1–25 (g)	22^{b}	16^{ab}	18^{ab}	15^{a}	16^{ab}
Total FI, d 2–26 (g)	147^{c}	138^{abc}	141^{bc}	125^{a}	128^{ab}
BW change/total FI	0.15^{b}	0.12^{ab}	0.13^{ab}	0.12^{ab}	0.13^{a}
Normalized FI (g diet consumed/d/g BW)[2]	0.056^{b}	0.055^{b}	0.056^{b}	0.049^{a}	0.051^{a}

[1] Values not sharing a superscript in common are significantly different at $p < 0.05$.

[2] Body weights were available between days 1–25 on 5 occasions; food intake was recorded daily. Daily food intake values were divided by body weight at the end of each weekly period to calculate an overall mean. BW, body weight; FI, food intake.

Table III: Lipid Blood Parameters after 4 weeks (mM in plasma)[1]

	Control	10% grain	20% grain	2.5% oil	5% oil
Total Cholesterol	7.7[b]	7.5[b]	7.1[ab]	7.1[ab]	6.5[a]
VLDL cholesterol	2.6[c]	2.1[bc]	1.4[ab]	1.3[a]	1.5[ab]
LDL cholesterol	2.8[a]	2.8[a]	2.8[a]	3.1[a]	2.9[a]
HDL cholesterol	2.3[ab]	2.8[c]	2.8[c]	2.6[bc]	2.2[a]
VLDL + LDL cholesterol	5.4[b]	4.8[ab]	4.3[a]	4.4[ab]	4.2[a]
VLDL + LDL)/HDL cholesterol	2.4[b]	1.8[a]	1.5[a]	1.7[a]	1.9[ab]
LDL/HDL cholesterol ratio	1.2[a]	1.0[a]	1.0[a]	1.2[a]	1.3[a]
TAG	3.3[ab]	3.8[b]	3.5[ab]	2.9[ab]	2.6[a]

[1] Values not sharing a superscript in common are significantly different at $p < 0.05$.

Table IV: Fecal Bile Acids and Neutral Sterols after 4 weeks (nmol/g body weight/day)[1]

	Control	20% Grain	5% Oil
Chenodeoxycholic	0.02[a]	ND[a]	0.1[a]
Cholate	4.7[a]	4.7[a]	4.2[a]
Deoxycholate	0.2[a]	0.2[a]	0.3[a]
Lithocholate	3.7[a]	4.0[a]	3.7[a]
Ursodeoxycholate	0.1[a]	0.3[b]	0.8[c]
Sum of identified fecal bile acids	8.7[a]	9.3[a]	9.2[a]
Cholestanol	7.2b	7.0[b]	4.9[a]
Cholesterol	7.2[a]	11.0[b]	15.0[c]
Coprostan-3-one	0.4[a]	0.5[b]	0.6[b]
Coprostanol	12.0[a]	15.0[a]	13.0[a]
Other sterols	59.0[a]	68.0[b]	145.0[c]
Total neutral sterols	86.0[a]	102.0[b]	178.0[c]

[1] Values not sharing a superscript in common are significantly different at $p < 0.05$.
ND, not detected.

both grain groups and 2.5% oil group relative to control. A similar trend existed for the two amaranth grain groups for LDL/HDL ratio ($p = 0.027$; Tukey's test failed to detect significant group differences). TAG was higher in the 10% grain group than in the 5% oil group.

Hamster cholesterol synthesis. FSR for control, 20% grain, and 5% oil groups were 0.0256, 0.0263, and 0.0322 percent per day, respectively. The 5% oil group had a significantly higher cholesterol synthesis rate than 20% grain and control groups, whether or not three outliers were excluded.

Fecal bile acids and neutral sterols. Total excreted fecal bile acids were unchanged by diet, however individual species changed (Table IV). Ursodeoxycholic acid excretion increased 2.5- and 6.0-fold in 20% grain and 5% oil groups, respectively, relative to control. Excretion of total neutral sterols, coprostan-3-one, and cholesterol was increased in 20% grain and 5% oil groups.

Hamster relative liver weights. Liver weights divided by body weights at time of dissection were 3.54%, 3.64%, 3.58%, 3.37%, and 3.33% for control, 10% grain, 20% grain, 2.5% oil, and 5% oil groups, respectively (average standard error, 0.06%). Both oil groups trended to have smaller relative liver weights relative to control ($p < 0.06$).

Liver esterified and unesterified cholesterol. In the 5% oil group, liver total and esterified cholesterol content/g liver was 15–17% lower, and unesterified:esterified cholesterol ratio higher, relative to control. On a whole liver basis, these differences were maintained since relative liver weight in the 5% oil group was reduced by only 1.06 ($p < 0.05$, Table V). Twenty-percent amaranth grain did not affect the above parameters. The values for µmoles of free and esterified cholesterol per gram of liver were in the same range as that obtained previously with hamsters fed diets containing 0.12% cholesterol and saturated fatty acids [21].

Discussion

Palatability and digestibility of amaranth. Food intakes and weight changes indicated hamsters consumed less of diets containing crude amaranth oil and were less efficient converting consumed calories to weight gain. Thus, amaranth oil may have been less palatable, and possibly less digestible. Possible anti-nutritional factors in amaranth include anti-enzyme and hemagglutinin [22], trypsin inhibitors, and lectins [23]. These heat-labile factors should have been destroyed by the heat produced during extrusion of the amaranth grain [1, 24, 25], from which amaranth oil was obtained. Lipid-soluble factors in the crude oil (pigments, waxes) could have affected digestibility. Our oil was not oxidized (which could affect palatability) since the peroxide value of 1.2 mEq O_2/kg was within acceptable crude oil limits.

Table V: Liver Cholesterol after 4 weeks (µmol/g liver)[1]

	Control	20% Grain	5% Oil
Unesterified cholesterol	13[a]	12[a]	13[a]
Esterified cholesterol	75[b]	77[b]	62[a]
Unesterified/Esterified cholesterol ratio	0.17[a]	0.16[a]	0.22[b]
Total cholesterol	88[b]	90[b]	75[a]

[1] Values not sharing a superscript in common are significantly different at $p < 0.05$.

Effects of amaranth grain and oil on cholesterol metabolism. Amaranth oil decreased total plasma cholesterol, whereas there was a trend for reduced LDL/HDL ratio (and higher HDL) in amaranth grain groups than in amaranth oil groups. Thus, oil and non-oil [fiber, saponin; 6, 8, 10, 26] amaranth components did not affect cholesterol metabolism identically. Grain components could have increased HDL, by various mechanisms including inhibition of cholesteryl-ester transfer protein [27].

Although VLDL cholesterol was decreased by amaranth grain and oils, a decrease in LDL cholesterol was not observed possibly because the level/type of saturated fat and cholesterol in the basal diets was sufficiently high to shut down LDL receptors [14, 28, 29] and increase direct secretion of LDL independent of VLDL [29]. Saturated fats (*e.g.,* 12:0 and 14:0) in the control diet could have acted synergistically with 0.12% dietary cholesterol to increase VLDL production and decrease VLDL remnant clearance [29]. In previous hamster experiments with 0.12% or higher levels of cholesterol and similar amounts and types of saturated fats, VLDL cholesterol was also increased [30–34], without LDL cholesterol changes [21, 35]. Amaranth may have acted to decrease VLDL cholesterol by decreasing VLDL secretion and upregulating VLDL receptors, leading to enhanced clearing. It is less likely amaranth lowered VLDL cholesterol by enhancing hepatic lipase-mediated conversion of VLDL to LDL; increased hepatic lipase activity is typically associated with reduced HDL cholesterol, which was not observed. High cholesterol feeding can also increase hepatic cholesterol levels and normalized liver weights [30]; both effects were reduced with 5% amaranth oil.

Similar to our reduction in total and VLDL cholesterol in hamsters, amaranth has been found to lower cholesterol in rats [6, 7, 9, 10] and chickens [8]. Experiments in chickens similarly showed amaranth grains and oils have cholesterol-lowering properties [8]. In contrast, a rat study showed that both whole and defatted amaranth grain equally lowered cholesterol, suggesting amaranth oil components might not have important cholesterol-lowering properties [9]. During grain de-fatting, some active non-saponifiable components may have remained with the grain, and/or amaranth processing and speciation [8], and experimental conditions (animal species, experimental diets) could have affected overall results [36].

Active components in amaranth: fatty acids. Fatty acids can affect VLDL and LDL cholesterol. It is not likely amaranth fatty acids had unique cholesterol-lowering properties [7] in the present experiment since control animals received corn oil with similar fatty acid composition (Table Ib; [15]). Corn and amaranth oils also have a similar positional distribution of fatty acids on the glycerol backbone [2, 15, 17]. Fatty acid positional distribution differences of our specific experimental oils were not evaluated. Differences in positional distribution, particularly of saturated fatty acids, can affect cholesterol-lowering efficacy [37, 38]. Fatty acids such as those found in fish oil are known to affect VLDL secretion.

Active components in amaranth: squalene. Amaranth oil contains 8–11% squalene [2, 17, 39]; amaranth oil with 8% squalene content was used in the present study. Dietary squalene has an overall neutral or variable effect on plasma cholesterol in hamsters [40] and humans [41, 42]. In humans, squalene was directly converted to cholesterol, increased tissue ACAT [41], and by increasing cholesterol synthesis, increased fecal excretions of cholesterol, and its derivatives, coprostanol, epicoprostanol, coprostanone, and bile acids [41]. In hamsters, 1% dietary squalene increased hepatic cholesterol and cholesterol ester, consistent with increased cholesterol synthesis and hepatic ACAT activity [40]. In our 5% amaranth oil group, hamsters received 0.4 weight-percent squalene and cholesterol synthesis was increased, but hepatic cholesterol ester was decreased. It is difficult to ascertain the role of squalene presently.

Active components in amaranth: plant sterols. There was evidence cholesterol absorption was inhibited by amaranth grain (20%) and oil (5%) since fecal cholesterol and total fecal sterols (including a large quantity of nonidentified sterols) were increased. The fecal bacterial product of cholesterol, coprostan-3-one, was also increased, but coprostanol was not. Plant sterols and fibers can also bind to bile acids and increase fecal bile acid levels [43]. Of the fecal bile acids examined, only ursodeoxycholate was increased by 20% grain, and particularly 5% amaranth oil. The fact that the 20% amaranth grain group, providing less plant sterols than the 5% oil diet, also influenced fecal sterols, is an indication that amaranth's fibrous and oil components impair cholesterol and bile acid absorption.

The 5% amaranth oil diets provided 0.12–0.13% non-cholesterol sterols (calculated from individual sterol analysis, Table Ic; or from the amount of sterols in amaranth oil). Amaranth oil (5%) may have increased the cholesterol synthesis rate via compensatory mechanisms following plant sterol-induced inhibition of cholesterol absorption, as reported in hamsters [44] and humans [45].

In other hamster experiments, the plant sterol dose to inhibit cholesterol absorption was 0.25–1.0% [36, 40, 44, 46, 47]. In hamsters, 0.25% plant sterols decreased total and esterified hepatic cholesterol levels without affecting fecal bile acids and bile acid synthesis [47]; 1% plant sterols decreased liver cholesterol and liver weight/body weight [46].

Amaranth plant sterols are unique in being very low in β-sitosterol, and very high in α-spinasterol [48]. Whether the high amount of α-spinasterol would impart special cholesterol-lowering characteristics is not known, but probably it behaves as do other 4-desmethyl sterols from soy and tall (pine wood) oils with respect to binding intestinal dietary and biliary cholesterol. A recent study [17] showed the predominant sterol (42%) in *A. cruentus* to be clerosterol [(24S)-24-ethylcholesta-5,25-dien-3 beta-ol] rather than α-spinasterol [(22E,24S)-24-ethyl-5 alpha-cholesta-7,22-dien-3 beta-ol; 62% in our analysis]. Our identification was based on earlier literature reports [48]. León-Camacho *et al* [17] also found much higher proportions of Δ^7-campesterol (25%), Δ^7-stigmastenol (15%), and Δ^5-avenasterol (12%) than our analysis indicated, and determined that 80% of the plant sterols were naturally esterified. They similarly found 2.5% total desmethyl sterols in amaranth oil, as compared to 2.7% in our analysis (Table Ic). Methyl sterols and terpenic alcohols were reported to be 0.26% in *A. cruentus* oil.

Active components in amaranth: tocopherols and tocotrienols. Tocotrienols are thought to possess cholesterol-lowering properties [49]. Our own unpublished analyses and two other reports [16, 17] have found, however, *A. cruentus* to be devoid of tocotrienols (as well as β-glucans) in contrast to one report [50]. *A. cruentus* is also not a rich source of α-tocopherol, but does have higher levels of β-tocopherol; however, the β-isoform is not a good *in vivo* antioxidant, and would not protect amaranth oil [51] nor lipoprotein particles from oxidation, nor have other effects attributed to α-tocopherol such as reductions in platelet adhesion and aggregation; changes in platelet morphology [52]; and modification of plaque enlargement [53].

Active components in amaranth: proteins. In the present experiment, amaranth protein could also have affected VLDL cholesterol. Amaranth protein replaced casein, and casein can raise apo B-containing lipoproteins in hamsters [54]. For example, replacement of casein with soy decreased VLDL secretion (or increased clearance) leading to decreased VLDL cholesterol levels without affecting LDL or total cholesterol [35].

In summary, we measured various cholesterol parameters (never before examined in a single amaranth experiment) following amaranth grain and oil feeding, and then evaluated which of amaranth's known chemical components could be responsible for the observed changes. There was evidence that amaranth decreased cholesterol absorption in a manner consistent with a plant sterol- and possibly fiber-mediated effect (*e.g.,* accumulation of some neutral and acidic fecal sterols). This then led to the observed decrease in normalized hepatic weight, hepatic cholesterol and cholesterol ester deposition, and the inferred reduction of VLDL cholesterol ester output leading to the observed reduction in VLDL cholesterol. The protein components of amaranth could also have contributed to any decreased VLDL particle secretion. The cholesterol synthesis rate was increased by 5% amaranth oil, likely due to compensatory mechanisms following the inhibition of cholesterol absorption. Fibrous components of amaranth grain could have been responsible for the observed increase in HDL. A concomitant decrease in LDL cholesterol was not observed with amaranth because the level/type of saturated fat and cholesterol in the basal diets was sufficiently high to shut down LDL receptors and increase direct secretion of LDL. Fatty acids, squalene, tocopherols, and tocotrienols were not likely to be responsible for the cholesterol-lowering effects observed in the present experiment.

Further investigations into the active cholesterol-lowering components in amaranth in hamsters are continuing [11]. A human pilot study assessing palatability prior to a potential, larger clinical study [13] has been completed with some promising results.

Acknowledgments

The authors wish to thank Christelle Piguet-Welsch, Isabelle Meirim, Pierre-Alain Golay, and Didier Gumy for their collaboration.

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Dr. Pierre Lambelet

Nestlé Research Center
P.O. Box 44
Vers-chez-Les-Blanc
1000 Lausanne 26
E-mail: pierre.lambelet@rdls.nestle.com