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ORIGINAL ARTICLE

Amaranth Oil Increased Fecal Excretion of Bile Acid but Had No Effect in Reducing Plasma Cholesterol in Hamsters

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Abstract Hamsters were fed for 4 weeks on four different diets: control (C) (balanced diet containing 20 % corn oil as the lipid source), hypercholesterolemic (H) (identical to C but containing 12 % coconut oil, 8 % corn oil and 0.1 % cholesterol as the lipid source), amaranth oil (A) (identical to H without corn oil but with amaranth oil), and squalene (S) (identical to H but admixed with squalene in the ratio found in amaranth oil). There were no significant differences in lipid profile, and in the cholesterol excreted in the animals' feces from amaranth oil (A) and squalene (S) groups. Fecal excretion of bile acids was greater in the amaranth oil (A) and squalene groups (S) as compared to the other groups. The scores of steatosis and parenchymal inflammation observed in the amaranth oil (A) and squalene groups (S) were superior to the ones observed in the other groups. Our findings demonstrated that amaranth oil, and its component squalene, increased the excretion of bile acids but did not have a hypocholesterolemic effect in hamsters fed on a diet containing high amounts of saturated fat and cholesterol.

Keywords Amaranth · Vegetable oil · Squalene · Lipid metabolism · Hamsters · Hypocholesterolemic diet

Abbreviations

C	Control diet group
H	Hypercholesterolemic diet group
A	Amaranth oil diet group
S	Squalene diet group
GC	Gas chromatography
HPLC	High performance liquid chromatography
FER	Food efficiency ratio
TC	Total plasma cholesterol
TG	Triacylglycerols
HDL	High density lipoprotein
LDL-C	Low density lipoprotein cholesterol
VLDL-C	Very low density lipoprotein cholesterol
UV	Ultra violet
vis	Visible
HE	Hematoxylin eosin
ANOVA	Analysis of variance
HMG-CoA reductase	Hydroxy methyl glutaryl coenzyme A reductase
ACAT	Acetyl coenzyme A acyltransferase

Introduction

Amaranth is a pseudo cereal of the class of dicotyledons, domesticated in America for over 6,000 years by the Incas,

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Mayans and Aztecs. Although there are more than 60 species of amaranth already identified, the most studied and consumed as food are *Amaranthus caudatus* L., *Amaranthus cruentus* L., and *Amaranthus hypochondriacus* L. [1]. Their proximal composition is similar and all present protein with a biological value comparable to protein from animal origin because the adequate amounts of essential amino acids, especially lysine, which is limited in cereals, and sulfur amino acids, limited in legumes. In addition, amaranth contains calcium, iron and zinc, and about 7 % lipids, the latter with more than 75 % of polyunsaturated fatty acids and isomers of vitamin E (tocopherols and tocotrienols) [2–6].

Several studies with amaranth grain in animals have shown its ability to reduce serum cholesterol levels [7–9]. These effects were also observed in human subjects [10]. Researchers have debated what substances would be responsible for the hypocholesterolemic property of amaranth. Some ascribe this ability to soluble dietary fiber [11], others to its protein [8, 9], and some others to squalene, a substance present in the lipid fraction of amaranth, which is an intermediate in the cholesterol synthesis pathway [12, 13].

A combination of mechanisms may be involved in the hypocholesterolemic effect of the amaranth grain. The reduction in plasma cholesterol can be achieved by diminished absorption of bile acid and cholesterol micelles, by inhibition of the cholesterol synthesis, by the increase in the expression of LDL receptors in the liver and by the modulation of the signaling paths that control cholesterol metabolism. Amaranth components that can favorably interact in these regulatory pathways are fiber, peptides derived from protein, phytosterols, unsaturated fatty acids and other phytochemicals with specific activity [9, 11–13]. Protein alone was found to have a hypocholesterolemic effect that responds to practically all cholesterol reductions observed with the whole grain [9] and also to prevent steatosis observed in hypercholesterolemic diets [14, 15]. Nevertheless, some reports in the literature attribute a major role to other components, specially the oil moiety, which is rich in squalene [16, 17].

Therefore, this study aimed to investigate the effect of amaranth oil, and its component squalene, on lipid metabolism in hamsters fed on a diet containing cholesterol and saturated fat.

Materials and Methods

Amaranth and Lipid Extraction

Initially, amaranth grain (*Amaranthus cruentus*) was subjected to a cold pressing process using a press (Model MPE-40, Ecirtec, Bauru, Brazil) in order to increase surface contact with the solvent. The next step was the direct extraction with *n*-hexane, in a pilot plant.

The residue after pressing was washed twice with the solvent at 50 °C, the first wash for 30 min and the second for 20 min. After the extraction, the solvent was evaporated at 70 °C, and the oil collected.

During this study, amaranth oil was stored at room temperature in amber glass vials with a nitrogen atmosphere to preserve the oil from light and air. The same procedure was performed to commercial refined corn oil (Liza, Cargill, Curitiba, Brazil).

Characterization of Amaranth and Corn Oils

Determination of Fatty Acids

Fatty acids were determined in triplicate, as methyl esters of fatty acids by gas chromatography (GC). An aliquot of 150 mg of lipid was taken and subjected to hydrolysis with 0.5 mol L^{-1} NaOH in methanol. It was then esterified with BF_3 in methanol with heating at 100 °C for 2 min in water bath [18].

Methyl esters were extracted by using 5 mL of cold *n*-hexane and kept refrigerated until injection into the gas chromatograph.

Thereafter, the methyl esters of the fatty acids in the lipid samples were separated and identified by gas chromatography, in a Shimadzu (Tokyo, Japan) apparatus model GC2010. The chromatographic conditions were: capillary column of fused silica Supelco (Bellefonte, USA) model SP-2560, 100 m long, 0.25 mm internal diameter and 0.20 μm in the film; hydrogen as a carrier gas was used at 1.0 mL min^{-1}; the splitter injector was set at 250 °C, with a split ratio of 1:20; the FID detector was set at 270 °C; column programming: initial temperature of 140 °C maintained for 5 min, a subsequent rise of 4 °C min^{-1} to 230 °C and 5 °C min^{-1} to 240 °C. The volume injected into the chromatograph of each extract was 1 μL, and identification was made by comparing the corrected retention time of methyl esters of fatty acids from samples and standards. The standard fatty acid methyl ester mixtures 47,885 Sigma-Aldrich (Bellefonte, USA) was used for identification of the peaks. The analysis was qualitative, and the proportion of each fatty acid was calculated by dividing the peak area by the total area of the run.

Determination of Squalene

Initially, the unsaponifiable fraction was isolated. An aliquot of 3 g of amaranth oil (or corn oil) was dissolved in a mixture of 30 mL of 95 % ethanol and 5 mL of 50 % KOH, and stirred for 1 h. The mixture was then washed with 40 mL of 95 % ethanol and diluted to 80 mL with

distilled water. The unsaponifiables were extracted five times using 50 mL of petroleum ether each time [19].

Analysis of squalene was performed by high-performance liquid chromatography (HPLC). Twenty microliters were injected and the mobile phase was a mixture of acetone/acetonitrile (40:60, v/v). Isocratic flow was used at 1 mL min^{-1}. Detection was performed by absorbance at 208 nm with an UV/vis detector (Shimadzu spectrophotometer, Tokyo, Japan); and the run time was 20 min [19]. Analyses were performed in triplicate. Squalene (3626, Sigma-Aldrich, Bellefonte, USA) was used as the external standard.

Determination of Sterols

The procedures described elsewhere [20, 21], with some modifications, were adopted. Initially, 0.3 g of the sample was admixed with 10 mL of ethanolic KOH (3 %) and heated in a water bath at 50 °C for 3 h. The sample was then cooled by adding 10 mL of distilled water, extracted four times with 10 mL of hexane and dried under N_2 flow. One microliter of the internal standard dehydrocholesterol (30800, Sigma-Aldrich, Bellefonte, USA) was added, and the sample injected into a gas chromatograph (HP model 6890, Santa Clara, USA). The equipment was fitted with an auto sampler; injector in split mode, ratio 1:50; HP-5MS capillary column (30 m, 0.25 mm id, 0.25 μm of film); flame ionization detector. The chromatographic conditions were optimized based on the methodology of Schmarr et al. [22], which are: column temperature programmed to initial temperature 150 °C for 0.1 min, increasing to 300 °C on a rate of 10 °C min^{-1}, and kept at this temperature for 10 min; drag gas helium at a flow rate of 1 mL min^{-1}; flame ionization detector: "make-up" gas, nitrogen at 30 mL min^{-1}, hydrogen 30 mL min^{-1}, synthetic air 300 mL min^{-1}, and temperature of 300 °C; injector temperature 250 °C.

Peaks were identified by comparing their retention times with the ones of β-sitosterol, 9889; and stigmasterol, 2424, Sigma-Aldrich, Bellefonte, USA), and they were quantified through the internal standard (dehydrocholesterol) area. Analyses were performed in triplicate.

Other Chemical Analyses

The acidity index was determined by the method Ca 5a-40; peroxide value by the method Cd 8-53; the refractive index according to the method Cc 7-25 in an Abbe Refractometer (40 °C) [23].

Animals and diets

The animals [male hamsters (*Mesocricetus auratus*), Golden Syrian strain, weanling (approximately 21 days), of sanitary standard type] were placed in individual cages containing retorted wood shavings, in a room with temperatures within 20–25 °C, and exposed to 12-h periods of light and darkness.

The animals had free access to water and the feed. They were weighed once a week, and cleaning of the cages was carried out three times per week.

The diets followed the nutritional recommendations of the National Research Council [24] and were formulated with a mixture of minerals and vitamins appropriate for hamsters' growth in accordance with the recommendations for rodents AIN-93 [25]. The composition of the diets is shown in Table 1.

The non-lipid components of the diets were acquired from Rhoster Ltd (Araçoiaba da Serra, Brazil) in powder form to be reconstituted with distilled water and oil. We chose to add the oils (amaranth, and corn plus squalene) in the laboratory, thus avoiding any oxidation during the industrial processes of feed manufacturing.

The oils were added to the diet powders, and the mixture was homogenized with a spoon. To each 100 g of the diet powder + oil, 50 mL of distilled water was added, and again, a homogenized mass was formed. This dough was then placed in ice forms and stored at −20 °C for about 4 h. The solid cubes formed were taken out and lyophilized for 4 h, and were then stored in plastic bags and at −4 °C until they were offered to the animals.

Diet consumption was monitored daily throughout the biological assay. The amount of feed intake was determined by the difference between the amount of feed supplied, and the amount left in the feeder.

Animals were weighed weekly, at the same time, in a digital semi analytical balance throughout the trial to evaluate the weight gain.

Table 1 Composition of diets used in the experiment (g kg^{-1})

Ingredients (g kg^{-1})	C	H	A	S
Casein	200	200	200	200
Amaranth oil	–	–	80	–
corn oil	200	80	–	–
Corn oil + squalene	–	–	–	80
Coconut fat	–	120	120	120
Cholesterol	–	1	1	1
Choline chlorine	2	2	2	2
Sucrose	100	100	100	100
Corn starch	350	349	349	349
Cellulose	100	100	100	100
L-Cystine	3	3	3	3
Mineral mix[a]	35	35	35	35
Vitamin mix[a]	10	10	10	10
Total	1000	1000	1000	1000

[a] Reeves et al. [25]

From the ratio between weight gain and amount of feed consumed, the food efficiency ratio (FER) was calculated for each diet offered.

Moisture, ash, fat and protein from the diets were determined according to methods 950.46, 923.03, 920.39C and 960.52 of the AOAC [26], respectively. All analyzes were performed in triplicate. The total content of carbohydrates was calculated by difference.

The biological experiment was conducted on 46 hamsters. After 7 days of adaptation and receiving a commercial diet, six animals were randomly selected and fasted about 8 h to collect blood for the analyzes in plasma as described below, and they were then killed.

The remaining animals were randomly divided into four groups of 10 each, receiving different types of diets, as described below.

- Control group (C) normal diet with 20 % corn oil as the lipid source;
- Hypercholesterolemic group (H): diet with 12 % coconut fat, 0.1 % cholesterol and 8 % corn oil as the lipid source;
- Amaranth oil group (A): hypercholesterolemic diet with 12 % coconut fat, 0.1 % cholesterol and 8 % of amaranth oil;
- Squalene group (S): hypercholesterolemic diet with 12 % coconut fat, 0.1 % cholesterol and 8 % of corn oil containing 4.6 % squalene (same amount of squalene in the oil amaranth).

After consumption of these diets for 28 days, all animals had their blood sampled by cardiac puncture to perform the analyses described below, and they were then killed by exsanguination.

This study protocol was approved by the Animal Ethics Committee, Instituto de Medicina Tropical (IMT/USP—027/2008), the Animal Ethics Committee, Faculdade de Ciências Farmacêuticas (FCF/USP—256) and the Environmental Ethics Committee, Faculdade de Saúde Pública (FSP/USP—1884), Universidade de São Paulo, Brazil.

Analyses in the Plasma

The animals were anesthetized in a CO_2 chamber for blood collection. Their blood was collected by cardiac puncture and placed in micro centrifuge tubes containing anticoagulant heparin (Liquemin), with a final concentration of approximately 1 mg mL^{-1}, and immediately subjected to low speed centrifugation (1,000×*g*, 15 min, 4 °C) to obtain the plasma [27].

The concentrations of total cholesterol (TC), triglyceride (TG) and high-density lipoprotein (HDL) were determined using the Cobas-Mira system (Roche Diagnostics, Indianapolis, USA).

The non-HDL cholesterol (LDL-C + VLDL-C) was calculated by subtracting HDL cholesterol from the total cholesterol.

Analyses in the Liver

The livers were weighed, and their average values were compared among groups.

Cholesterol The hepatic cholesterol concentration was measured using the method proposed by Katsanidis and Addis [28], with some modifications.

Seven hundred milligrams of liver sample were homogenized in a saponification solution [11 % KOH in a 55:45 ethanol/water (v/v) mixture]. The sample was placed in a water bath for 15 min at 80 °C. After cooling and partition, the organic phase was used.

Quantification of cholesterol was performed by HPLC on an SCL-10A apparatus (Shimadzu, Tokyo, Japan). The injection volume was 20 μL, and the mobile phase was *n*-hexane/isopropanol (97:3 v/v), with an isocratic flow rate of 1 mL min^{-1}. The detector used was a UV/VIS diode array scanning with the reading at a wavelength of 206 nm. Quantification was performed using a calibration curve of standard cholesterol (Steraloids, Wilton, USA). The run time was 8 min, and analyses were performed in triplicate [29].

The efficiency of the method used was tested by the recovery of added cholesterol using a cholesterol standard.

Histological Analysis After sacrifice, parts of the animal livers were removed and preserved in 10 % buffered formalin for at least 48 h and cross-sections were made of these organs.

The sections were submitted to routine histological techniques with inclusion in paraffin to obtain sections 5 μm thick, and stained with hematoxylin-eosin (HE).

Histological analysis was performed by the staff of the Laboratory of Pathology, School of Medicine, University of São Paulo. For the morphometric study, we used an ordinary optical microscope (Nikon Eclipse E200, Otawara, Japan) coupled to a digital color camera (SCC-131, Samsung, Ridgefield Park, USA).

Analyses in the Feces

The animal feces were individually collected during the last 5 days of the experiment. The animals were housed in cages specially built for this purpose.

Feces were weighed and packed in polyethylene bottles, frozen and lyophilized for 72 h. After drying, they were weighed again, crushed and stored at −20 °C until the moment of analyses.

For the cholesterol analysis, 50-mg aliquots of stool were subjected to saponification with 700 μL of methanol and 200 μL of 5 mol L^{-1} NaOH solution for 2 h in a water bath under stirring at 80 °C. After addition of saturated NaCl solution, the cholesterol was extracted three times with 3 mL of petroleum ether [30].

The sample was dried and resuspended in 800 μL *n*-hexane HPLC grade. Quantification was performed using HPLC equipment—SCL-10A (Shimadzu, Tokyo, Japan)—with a UV/vis diode array detector, a manual Rheodyne injector and a quaternary pump. The injection volume was 20 μL. The mobile phase was a mixture of *n*-hexane/isopropanol (97:3 v/v) with an isocratic flow of 1 mL min^{-1}, and the stationary phase was composed of silica. The detection wavelength used was 206 nm. The run time was 8 min [30].

Bile acids were quantified using a commercial kit brand Diazyme (San Diego, USA). Previously, feces were extracted with 50 % *tert*-butanol in water for 15 min at 37 °C and centrifuged at 10,000×*g* for 2 min to obtain the supernatant containing the bile acids [31].

Statistical Analysis

The results were expressed as means with standard deviation or standard error. To determine the mean difference between two variables, we used the Student *t* test. Analysis of variance (ANOVA) followed by Tukey's test (when a difference between groups was detected) were performed to compare the averages of three or more samples.

The Statistical Package for Social Sciences (SPSS, New York, USA) was used, and the means were considered different at the 5 % significance level.

Results

The profile of the major fatty acids of amaranth and corn oils is shown in Table 2.

As can be seen, both amaranth and corn oils exhibit similar fatty acid profiles, namely palmitic ($C_{16:0}$), oleic ($C_{18:1}$), and linoleic ($C_{18:2}$) being the most abundant fatty acids in both oils. The fatty acid α-linolenic acid ($C_{18:3}$) is present in small quantities in these oils (0.5 % in oil amaranth and 1.1 % corn oil).

The concentrations of phytosterols (campesterol, stigmasterol and β-sitosterol) and squalene of the amaranth and corn oils (mg 100 g^{-1}) are shown in Table 3.

In the present study, squalene represents 4.63 % of amaranth oil, which makes this oil an attractive source of this isoprenoid compound. In the *A. cruentus* species, Becker et al. [2] found 4.6 % of squalene in amaranth oil. He and Corke [6] determined the squalene content of 104 genotypes of 30 species of amaranth, finding an average concentration of 4.2 %, a value very close to the one observed in our study.

Table 2 Profile of the major fatty acids (%) of amaranth and corn oils, used in the diets of hamsters

Fatty acids (%)	Amaranth oil	Corn oil
$C_{14:0}$ (myristic)	0.1	0.0
$C_{16:0}$ (palmitic)	17.9	11.4
$C_{16:1}$ (palmitoleic)	0.5	0.1
$C_{17:0}$ (margaric)	0.1	0.1
$C_{18:0}$ (stearic)	3.8	2.3
$C_{18:1}$ (oleic)	36.0	38.4
$C_{18:2t}$ (linoleic *trans*)	0.5	0.3
$C_{18:2}$ (linoleic)	37.8	44.6
$C_{20:0}$ (arachidic)	1.0	0.7
$C_{20:1}$ (eicosenoic)	0.5	0.5
$C_{18:3}$ (α-linolenic)	0.6	1.1
$C_{22:0}$ (behenic)	0.5	0.2
$C_{22:2}$ (docosadienoic)	0.1	0.0
$C_{24:0}$ (lignoceric)	0.6	0.3
$C_{20:5}$ (timnodonic)	0.1	0.0
Totals		
Saturated	24.0	15.0
Unsaturated	76.0	84.7
Monounsaturated	37.0	39.0
Polyunsaturated	39.0	45.7

All experimental diets (Table 1) had 20 % lipids, which is the maximum of the nutritional needs of hamsters in the growth phase [24]. The quantity of amaranth oil used in the diet of the amaranth oil group was 8 %. The compositions of the diets of the four experimental groups are shown in Table 4, from which we can see that control (C), hypercholesterolemic (H), amaranth oil (A), and squalene (S) diets present composition that did not differ (ANOVA, $p < 0.05$).

Table 3 Phytosterols (campesterol, stigmasterol and β-sitosterol) and squalene levels in amaranth and corn oils (mg 100 g^{-1})

Component (mg/100 g)	Amaranth oil	Corn oil
Campesterol	13.44 ± 0.88[a]	99.52 ± 2.22[b]
Stigmasterol	17.92 ± 0.40[a]	41.58 ± 1.09[b]
β-Sitosterol	683.40 ± 19.60[a]	582.41 ± 5.42[b]
Total phytosterols[a]	714.77	723.50
Squalene	4.63 ± 0.18	Nd[b]

Different letters in the same rows indicate different means (*t*-Student test $p < 0.05$)

[a] Total phytosterols = campesterol + stigmasterol + β-sitosterol

[b] Not detected

The plasma lipid profiles for the hamsters at the baseline (after seven-day control diet adaptation), and after 28 days of consumption of the experimental diets, are presented in Table 5.

Total cholesterol concentrations in the plasma of groups A, S and H were higher than the observed in the group C. There was no difference in total cholesterol among groups A, S and H. The same results were found for triacylglycerols concentration, indicating that either amaranth oil or squalene are unable to reduce cholesterol and triacylglycerols in the plasma of the animals. The mean of the values of HDL cholesterol and non-HDL cholesterol were equal for groups A and S, which in turn were higher than the values observed for the control group.

There were no significant differences in liver weight adjusted by body weight among groups H, A and S. However, the control group presented lower values as compared to all the other groups, indicating that the fat profile influenced this parameter. There was also no difference in cholesterol content in livers of the groups H, A and S.

Characteristic liver steatosis and inflammation are shown in Fig. 1. Liver weights, cholesterol contents, degree of steatosis, excretion of bile acids and cholesterol in the feces after 28 days on the diets are presented in Table 6.

Hamsters fed on control and squalene diets presented a focal steatosis (score 1+). The amaranth oil group presented a more pronounced steatosis with more than 50 % of the center lobular veins affected (score 2+), the highest steatosis score among all groups followed second by the squalene group.

There was no difference in fecal excretion of cholesterol among the groups. The control group presented similar results as compared to the ones that received a hypocholesterolemic diet. Nevertheless, a significant increase in fecal excretion of bile acids was observed for amaranth oil and squalene groups as compared to the control and the hypercholesterolemic groups.

Discussion

The fatty acids' profile observed is similar, and within varietal differences to the ones reported in literature [32, 33]. The fatty acid profile of five varieties of amaranth showed the following variation: linoleic acid, 34.9–49.1 %, oleic 22.8–31.5 % and palmitic acid 21.4–23.8 %. Unsaturated

Table 4 Proximate composition of diets of the groups control (C), hypercholesterolemic (H), amaranth oil (A) and squalene (S) [g 100 g^{-1} diet (d.s.b.)]

	Groups			
	C	H	A	S
Ashes	2.67 ± 0.01[a]	2.54 ± 0.07[a]	2.20 ± 0.08[a]	2.46 ± 0.02[a]
Protein*	19.44 ± 0.63[a]	19.20 ± 1.05[a]	20.12 ± 0.25[a]	19.05 ± 0.37[a]
Lipids	20.91 ± 0.04[a]	20.26 ± 0.11[a]	20.50 ± 0.06[a]	20.54[a] ± 0.21[a]
Carbohydrate**	56.98	58.00	57.18	58.05
Total	100	100	100	100

Same letters in the same row indicate means did not differ, ANOVA, Tukey test ($p \geq 0.05$). Mean ± SD

C control, *H* hypercholesterolemic, *A* amaranth oil, *S* squalene

* N conversion factor = 6.25

** Calculated by difference

Table 5 Plasma lipid profile for the hamsters at the baseline (after seven-day control diet adaptation) and after 28 days of consumption of the experimental diets

	Baseline	C	H	A	S
Total cholesterol	72.67 ± 7.14	92.50 ± 6.86[a]	147.00 ± 12.58[b]	172.00 ± 19.45[b]	177.10 ± 15.24[b]
Triacylglycerol	87.00 ± 12.06	72.70 ± 4.43[a]	112.00 ± 7.00[b]	121.90 ± 9.48[b]	111.70 ± 5.46[b]
HDL-c	23.50 ± 4.31	37.60 ± 2.12[a]	50.50 ± 4.12[ab]	53.40 ± 4.64[b]	58.20 ± 3.52[b]
Non-HDL-c	49.17 ± 4.71	54.90 ± 5.38[a]	96.50 ± 9.02[ab]	118.60 ± 17.07[b]	118.90 ± 12.27[b]

Different letters at the same row indicate significant difference, ANOVA, Tukey test ($p < 0.05$). Comparison made for groups: control, hypercholesterolemic, amaranth oil, and squalene. Results are means ± SE

C control, *H* hypercholesterolemic, *A* amaranth oil, *S* squalene, *Non-HDL-c* Non-HDL cholesterol (VLDL + LDL) = Total Cholesterol − HDL cholesterol

fatty in these varieties ranged from 71.4 to 73.2 %, values very similar to those shown in Table 2 (77.2 %).

Phytosterols are naturally present in vegetables, particularly in seeds, vegetable oils, cereals, nuts and fruits [34]. Although more than 250 phytosterols have been already identified in nature, campesterol, β-sitosterol, stigmasterol and β-sitostanol are the major ones found in most of the foods [35]. The most abundant phytosterol in both oils is the β-sitosterol, and its content is higher in amaranth oil (683.40 mg 100 g^{-1}) than in corn oil (582.41 mg 100 g^{-1}). Campesterol and stigmasterol are present in greater amounts in corn oil (99.51 and 41.58 mg 100 g^{-1}, respectively) as compared to amaranth oil (13.44 and 17.93 mg 100 g^{-1}, respectively). However, the total content of phytosterols (stigmasterol + campesterol + β-sitosterol) was similar in both oils. Marcone et al. [36] determined the phytosterol content of four varieties of amaranth and in all of them, the β-sitosterol was again the most abundant. According to these authors, the content of phytosterols in amaranth oil is higher than that found in soybean oil, olive, peanut, coconut and palm. Almeida [21] determined the content of phytosterols from soybean oil, corn, canola, sunflower, olive oil and extra-virgin olive oil. For all these materials, the β-sitosterol was found in higher concentrations, whereas stigmasterol had the lowest concentrations. In this study, the highest content of total phytosterols was found in corn oil (803.09 mg 100 g^{-1}), followed by canola oil (646.48 mg 100 g^{-1}).

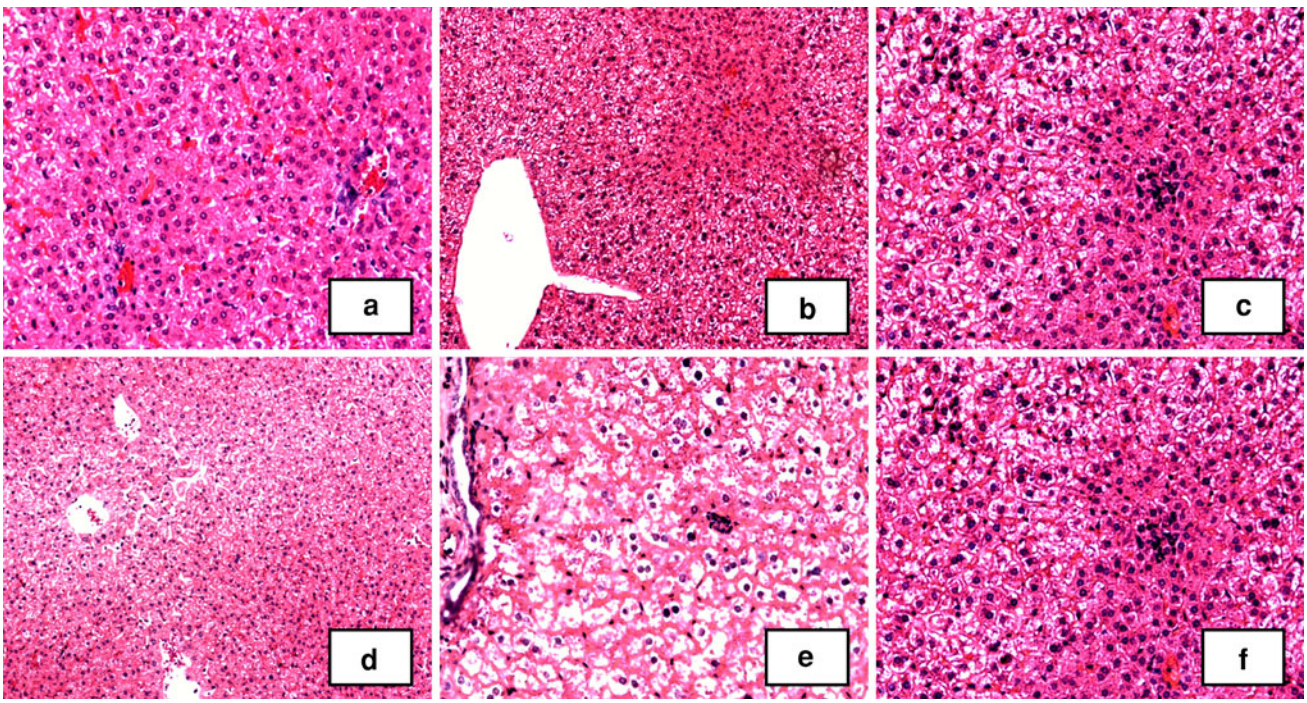

Fig. 1 Photomicrograph of sections 5 μm thick HE-stained liver illustrating: **a** the focal hepatic fat accumulation in the control group, **b** the diffuse hepatic fat accumulation (grade 3+) in the amaranth oil group, **c** the parenchymal inflammation (grade 1+) in the amaranth oil group, **d** the fatty steatosis (grade 2+) in the squalene group, **e** the parenchymal inflammation (grade 1+) in the squalene group, and **f** the process of apoptosis in the amaranth oil group

Table 6 Liver weight, cholesterol in the liver, degree of steatosis, excretion of bile acids and cholesterol in the feces, after 28 days of feeding the diets

	C	H	A	S
Liver weight (g 100 g^{-1})	4.12 ± 0.14^{a}	4.98 ± 0.11^{b}	5.08 ± 0.15^{b}	5.25 ± 0.16^{b}
Cholesterol in the liver (g 100 g^{-1} liver)	0.20 ± 0.01^{a}	0.22 ± 0.01^{ab}	0.22 ± 0.02^{ab}	0.28 ± 0.02^{b}
Degree of steatosis (0–4)	1.09 ± 0.07^{b}	0.08 ± 0.01^{a}	2.79 ± 0.08^{c}	1.58 ± 0.12^{b}
Bile acids (μmol g^{-1} feces)	1.30 ± 0.10^{a}	1.58 ± 0.11^{a}	2.24 ± 0.17^{b}	2.23 ± 0.08^{b}
Cholesterol in the feces (g 100 g^{-1} feces)	0.61 ± 0.01^{a}	0.61 ± 0.04^{a}	0.57 ± 0.10^{a}	0.39 ± 0.12^{a}

Mean ± SE, of three analyses. Degree of steatosis as follows: [0—no steatosis; 1—focal steatosis (less of 50 % of the center globular veins); 2—steatosis in excess of 50 % of the center globular veins; 3—diffuse steatosis; 4—diffuse and intense steatosis. The same superscript letters in the same row indicate no significant difference (ANOVA, Tukey, $p < 0.05$)]

Squalene was not detected in the corn oil used in this study. Tuberoso et al. [37] analyzed the content of squalene of various commercial vegetable oils, including corn oil and found only 0.03 % squalene in the oil. This component is affected by the refining processes and may occur in very low concentration, being sometimes not detected by the routine analytical procedures, as in our case. According to the literature, the amount of squalene in important vegetable oils such as olive oil, corn and sunflower ranges from 0.01 to 0.4 %, which is not sufficient to consider these vegetable oils sources of this compound [16].

With regard to the quality for human nutrition, the oil produced in this study should be refined to reduce the levels of acidity and peroxide (to <4 mg KOH g^{-1} and <15 mequiv kg^{-1}, respectively), according to the Brazilian legislation [37]. The corn oil presented 0.3 mg KOH/g in acidity index analysis and 2.2 mequiv kg^{-1} in peroxide analysis, adequate for human consumption [38]. The refraction index at 40 °C was 1.47 for both oils.

No significant difference was found between the total feed intake, average daily weight and final weights of the animals in all groups. The weight gain of the animals of group A was lower than the observed in groups C and H, but did not differ from the group S (Table 7), reflecting in the food efficiency ratio (FER) of the animals that consumed amaranth oil. The observed FER for group A was lower than the one observed for groups C and H, but did not differ from the group S.

Berger et al. [39] found a lower weight gain in the group of hamsters that received amaranth oil, extracted by organic solvent, as compared to the control group. They suggested that this fact can be associated with the presence of waxes and certain pigments in crude amaranth oil, which can only be removed by the refining process. These compounds may have resulted in lower digestibility of the amaranth oil compared to commercial corn oil used in the control group.

This low bioavailability of the amaranth oil as compared to the corn oil made the need of higher diet ingestion by these groups, lowering the Food Efficiency Ratio (FER). However, the final weights of the animals of all groups were the same not affecting their lipid metabolism in terms of plasma cholesterol (total, HDL-c, and non-HDL-c) and triacylglycerols [40]. Moreover, although that weight gain was lower, the animals grew normally to the same size and weight and were healthy throughout the experiment, according to usual parameters evaluated periodically such as bright eyes, soft and flawless fur, quick reflexes and absence of diarrhea or changes in behavior.

Berger et al. [39] offered amaranth oil (*A. cruentus*) to hamsters at 2.5 and 5.0 % concentrations in a hypercholesterolemic diet. They observe no reduction in total cholesterol of the animals that received 2.5 % of amaranth oil as compared to the control group, which received 2.5 % of commercial corn oil. However, the animals from the group that received 5.0 % of amaranth oil presented a reduction of 15 % in total cholesterol and 22 % in non-HDL cholesterol as compared to control group, which received 5.0 % of commercial corn oil. They concluded that hypocholesterolemic effect of amaranth oil is dependent upon the quantity of oil used. They proposed that there is an ideal concentration of amaranth oil and diet conditions to promote reduction in plasma cholesterol concentration. As our main objective was to identify the contribution of squalene to lipid metabolism, we used the maximum oil concentration in the diet recommended for hamsters (8 %), which resulted in about 0.4 % of squalene in the diet. Although the oil concentration we used was higher, the metabolic lipid response that we observed differed from that reported in the literature [39]. We observed no reducing effects at 8 % concentration of amaranth oil of in the diets. If there is an ideal concentration for the hypocholesterolemic effect of the amaranth oil, it is within 5–8 % in the diet.

The control group presented a lower content of cholesterol in liver compared to group S, but results were similar to groups H and A. Zhang et al. [41], likewise, found a higher concentration of cholesterol in the liver of hamsters

Table 7 Initial weight, final weight, weight gain, total feed ingestion, average daily consumption, and food efficiency ratio (FER)

	C	H	A	S
Initial weight (g)	94.1 ± 3.4[a]	94.8 ± 3.5[a]	99.7 ± 3.3[a]	98.6 ± 2.4[a]
Final weight (g)	139.7 ± 4.6[a]	139.0 ± 2.8[a]	130.8 ± 4.2[a]	136.9 ± 4.1[a]
Total feed ingestion (g)	265.1 ± 6.3[a]	276.3 ± 9.7[a]	259.9 ± 6.2[a]	261.3 ± 8.1[a]
Average daily feed consumption (g)	9.6 ± 0.2[a]	10.0 ± 0.3[a]	9.5 ± 0.2[a]	9.5 ± 0.3[a]
Weight gain (g)	45.6 ± 3.0[a]	44.2 ± 2.9[a]	31.2 ± 2.1[b]	38.4 ± 3.0[ab]
FER (%)	17.1 ± 0.9[a]	16.0 ± 1.0[a]	12.0 ± 0.7[b]	14.6 ± 0.8[ab]

FER (%) = [(Weight gain)/(total feed ingestion)] × 100

Different letters in the same row indicate significant differences (ANOVA, Tukey test, $p < 0.05$). Results are means ± SE

C control, *H* hypercholesterolemic, *A* amaranth oil, *S* squalene

that consumed a diet containing 0.05, 0.1 and 0.5 % of squalene as compared to the control group. The authors attributed this effect to the conversion of squalene to cholesterol in the liver. This conversion should increase cholesterol concentration in plasma, as we observed in the present study. A consequence of this elevated cholesterol concentration in plasma is the steatosis and inflammation of the liver.

Surprisingly, the hypercholesterolemic group presented the lowest degree of steatosis. However, this group was the one with the lower cholesterol concentration, which is, besides triacylglycerols, one of the determinants of this effect. Ronis et al. [42] using a hepatotoxicity model induced by alcohol verified lower concentrations of triacylglycerols and free fatty acids in rats fed a diet containing 30 % of saturated fat compared to animals that consumed one containing 30 % of corn oil. The authors did not verify the appearance of steatosis in any animal that received a diet rich in saturated fatty acids. Steatosis in the liver is caused mainly by the accumulation of triacylglycerol. Therefore, the reduction in the synthesis of fatty acids and the increase of its degradation could contribute to the absence of steatosis in the group that consumed a great quantity of saturated fatty acids. Fat accumulation in the liver of the animals from the C group when compared to the H group could be due to the fact that unsaturated fat is better absorbed than saturated fat, as verified by some authors [43, 44].

Tilvis and Miettinen [45] verified that the intestine of the rat presents a great capacity to absorb the squalene from the diet, and squalene absorbed is mainly converted to bile acids in the liver. Nakamura et al. [46] found that a part of the squalene metabolized to bile acids by the cholesterol pathway in hepatocytes is excreted in feces.

In humans, the study of Strandberg et al. [47] showed an increase in cholesterol and bile acids excretion after the consumption of squalene. They observed no increase in serum cholesterol, as we did in our study, probably due to compensatory mechanisms, such as the inhibition of the activities of HMG-CoA reductase (HMGR) and Acetyl-Coenzyme A acetyltransferase (ACAT) enzymes, and as a result of the increase of fecal excretion of sterols.

From our results, it can be clearly said that at the ingestion level studied, squalene, either present in the amaranth oil or admixed with corn oil, has a stimulating effect on cholesterol synthesis, due to its presence in the cholesterol synthesis pathway. This increase is followed by cholesterol incorporation into bile acids in a compensatory homeostatic mechanism, resulting in an increase in sterol excretion in the feces. If amaranth oil presents any hypocholesterolemic effect, it is observed at other concentrations, and it is not caused by squalene.

The consumption of amaranth oil and squalene promoted an increase in fecal excretion of bile acids, but no reduction in blood cholesterol of hamsters fed on a diet containing high quantities of cholesterol and saturated fatty acids. The highest degrees of steatosis were verified in animals fed with amaranth oil and corn oil admixed with squalene, which suggests that squalene plays an important role in the deposition of lipids in the liver.

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