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ORIGINAL PAPER

Biochemical and Molecular Study of the Influence of *Amaranthus hypochondriacus* Flour on Serum and Liver Lipids in Rats Treated with Ethanol

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Abstract Hyperlipidemia and hepatic steatosis are frequent alterations due to alcohol abuse. Amaranth is a pseudocereal with hypolipidemic potential among other nutraceutical actions. Here we study the effect of *Amaranthus hypochondriacus (Ah)* seeds on serum and liver lipids, and the expression of genes associated to lipid metabolism and liver histology in male *Wistar* rats intoxicated with ethanol. The animals were divided into four groups; two groups were fed the American Institute of Nutrition 1993 for maintenance diet (AIN-93M), and the other two with AIN-93M containing *Ah* as protein source. One of each protein group received 20 % ethanol in the drinking water, thus obtaining: CC (control casein), EC (ethanol casein), C*Ah* (control *Ah*) and E*Ah* (ethanol *Ah*). When comparing E*Ah vs*. EC, we found a positive effect of *Ah* on lipids, preventing the increment of serum cholesterol ($p<0.001$), through the higher expression of the LDL receptor ($p<0.001$); and it also decreased free ($p<0.05$) and esterified cholesterol ($p<0.01$) in liver, probably via the reduction of the 3-hydroxy-3-methylglutaryl coenzyme A reductase expression ($p<0.001$). We also observed that amaranth contributed to the decrease of fat deposits in liver, probably through the decrease in acetyl-CoA carboxylase alpha ($p<0.01$), glycerol-3-phosphate acyltransferase 1 ($p<0.01$) and diacylglycerol O-acyltransferase 2 ($p<0.05$) expression. The histological study showed a decrease in the fat deposits in the amaranth group when compared to casein; this is consistent with the biochemical and molecular parameters studied in this work. In conclusion, amaranth could be recommended to avoid the alterations in the lipid metabolism induced by alcohol and other harmful agents.

Keywords *Amaranthus hypochondriacus* · Ethanol · Cholesterol · Triglycerides

Abbreviations

ACCα	Acetyl-CoA carboxylase alpha
Ah	*Amaranthus hypochondriacus*
AIN-93M	American Institute of Nutrition 1993 for maintenance diet
Apo B	Apolipotrotein B
BHT	Butylated hydroxytoluene
C*Ah*	Control *Ah*
CC	Control casein
CPT-1	Carnitine palmitoil transferase 1
CYP7A1	Cholesterol 7-alpha-hydroxylase
DGAT-2	Diacylglycerol O-acyltransferase 2
E*Ah*	Ethanol *Ah*
EC	Ethanol casein
GPAT-1	Glycerol-3-phosphate acyltransferase 1
HDL-C	High-density lipoprotein-cholesterol
HMG-CoAR	3-hydroxy-3-methylglutaryl coenzyme A reductase
LDL-C	Low-density lipoprotein-cholesterol
PPARα	Peroxisome proliferator-activated receptor alpha
RT-PCR	Reverse transcription polymerase chain reaction
SREBP-2	Sterol regulatory element binding protein 2
TLC	Thin layer chromatography
Total-C	Total cholesterol
VLDL	Very-low-density lipoprotein

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Introduction

Alcohol abuse affects all the socioeconomic levels in the world, generating serious problems in public health. Even though several studies have shown that a moderate intake of alcohol decreases the risk for cardiovascular diseases [1, 2], the chronic and excessive ingestion of ethanol induces changes in lipid metabolism, predisposing to the development of steatosis, hyperlipidemia and atherosclerosis [3]. On the other hand, it is important to remark that a healthy eating pattern such as the Mediterranean diet, consistent in whole grains, fruits and vegetables, is shown to be efficient in reducing cardiovascular risk and in improving the lipid profile [4].

Amaranth is a pseudocereal from the *Amaranthaceae* family that is grown in a wide range of agricultural and weather conditions. This small seed is an important source of vegetable protein, rich in methionine and lysine amino acids, being this last one a limiting in many grains. The grain has high levels of soluble fibers, magnesium, calcium, tocotrienols and unsaturated fatty acids, especially linoleic acid [5, 6]; there have also been reports about significant levels of squalene, an important precursor of all the steroids [7]. Besides its nutritional quality, this grain exerts several nutraceutical actions due to the presence of a series of phytochemical compounds with hypolipidemic action [8–11] and antihypertensive potential [12], as well as antioxidant capacity [13–15]. Amaranth is also a gluten free grain, so it can be incorporated in the diet of celiac patients and those allergic to cereals [16].

The aim of this work was to evaluate the *Amaranthus hypochondriacus* effects on cholesterol and triglycerides metabolism in the liver of rats that were sub-chronically treated with ethanol. To this end, we determined serum and liver lipids and the expression of genes related; in addition, a histological study was performed.

Materials and Methods

Plant Materials

Amaranthus hypochondriacus (Ah) var. Antorcha was provided by the Department of Agronomy at the National University of Río Cuarto, Argentina, from a 2009 harvest of an experimental cultivation. For the flour preparation, the dried whole grain was ground and sieved through a 200 μm nylon sieve.

Animals and Experimental Design

Twenty-four male *Wistar* rats weighing 200 g were used. They were divided into four groups of six rats each, housed in individual cages and maintained under a regular light–dark cycle, with free access to food and water. All groups were fed according to recommendations of the American Institute of Nutrition 1993 (AIN-93M) for four weeks [17]. Two groups were fed with the AIN-93M diet and the other two with AIN-93 M containing *Ah* as protein source. One of each group received 20 % ethanol in the drinking water [18], thus obtaining: CC (control casein), EC (ethanol casein), C*Ah* (control *Ah*) and E*Ah* (ethanol *Ah*). An intake between 4 and 6 g/kg/day of alcohol per rat was estimated. All studies involving experimental animals were conducted in accordance to national and institutional guidelines for the protection of animal welfare. The study was approved by the Animal Care Committee of the National University of San Luis, Argentina.

Serum Parameters

Total cholesterol (total-C), HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) [19] were determined by enzymatic methods, using commercial kits (Wiener, Laboratorios S.A.I.C., Rosario Argentina). Proteins were determined by the Biuret method [20] with bovine serum albumin as standard.

Hepatic Lipids Determinations

Lipids were extracted from liver tissue [21]. Total lipids were determined by dry weight [22]. Lipids were resuspended in hexane and total cholesterol was determined using an aliquot [23, 24]. And other aliquots of the extracts were used for separating the different lipids on thin-layer chromatography (TLC). The lipids were detected by exposing the plates to iodine vapors; they were scraped off and were used for the determination of triglycerides [25] and free and esterified cholesterol [23]. Proteins were determined by the Biuret method [20].

Analysis of mRNA Expression by RT-PCR

The relative mRNA quantities of the genes coding for hydroxy-methylglutaryl coenzyme A reductase (HMG-CoAR), LDL receptor (LDLr), sterol regulatory element binding protein 2 (SREBP-2), cholesterol 7-alpha-hydroxylase (CYP7A1), apolipotrotein B (ApoB), glycerol-3-phosphate acyltransferase 1 (GPAT-1), diacylglycerol O-acyltransferase 2 (DGAT-2); acetyl-CoA carboxylase alpha (ACCα), peroxisome proliferator-activated receptor alpha (PPARα) and carnitine palmitoil transferase 1 (CPT-1), were determined by reverse transcription polymerase chain reaction (RT-PCR). The sequences of the different primers and sizes of the PCR products are shown in Table S1.

The PCR products were analyzed and photographed. The intensity of each band was measured using the NIH Image software Scion. The relative abundance of each band was then normalized according to the housekeeping gene β-actin, calculated as the ratio of the intensity values of each product to that of β-actin, and it was reported in arbitrary units (AU).

Morphological Study

A histological study of the liver was performed by light microscopy. Sections were stained with hematoxylin and eosin, and photographs were taken using a Leitz Dialux optical microscope supplied with a Leica camera (100×).

Statistical Analysis

Results are expressed as mean ± standard deviations. Statistical differences were tested by using a one-way analysis of variance (ANOVA). A probability of 0.05 or less indicates significant difference [26].

Results and Discussion

The aim of this study was to elucidate the possible effects of whole amaranth flour on the lipids content, using an experimental model of *Wistar* rats treated with ethanol (20 % ethanol solution) for four weeks. Serum and liver lipids were measured, as well as some genes involved in the lipid metabolism. In addition, a histological study of the liver was performed. We found that in presence of ethanol, *Ah* flour showed a beneficial effect on these parameters.

Table 1 shows that serum total-C and the lipoprotein fraction LDL + VLDL-C decreased in E*Ah vs*. EC, as well as in C*Ah vs* CC ($p<0.001$); while there were no significant differences in HDL-C. In addition, EC showed the lowest value in the HDL/total cholesterol ratio when compared to the other groups. These results suggest susceptibility of EC to the harmful action of ethanol, and also the protective effect of amaranth in E*Ah*.

Table 2 indicates that in EC *vs.* CC, the cholesterol and triglycerides liver content increased ($p<0.05$) and ($p<0.01$), respectively; free and esterified cholesterol also increased ($p<0.05$) and ($p<0.01$), respectively. In E*Ah*, when compared to EC, there was a trend of decreasing cholesterol (13.7 %) and triglycerides (11.5 %); also a decrease of free ($p<0.05$) and esterified ($p<0.01$) cholesterol was observed. From these data we infer that in the groups exposed to ethanol, amaranth would have a beneficial role in reducing the accumulation of lipids in the liver.

Since ethanol is metabolized in the liver and its effect on the lipid profile is complex, the more frequent alterations are reflected in the levels of lipoproteins and in the hepatic steatosis. The information available on alcohol impact on lipids is very controversial. In general, alcohol intake is frequently associated to an increase of HDL-C in blood and esterified cholesterol in liver [27]. Moreover, it is known that ethanol inhibits the release of VLDL particles, predisposing the development of fat liver [3]. All the modifications induced by ethanol can be modulated by molecular, genetic and environmental mechanisms; in addition, the patient factors are determinant as well as the multiple interactions of ethanol with the living organism.

Several components of grains have the ability to reverse some alterations in lipid metabolism. It has been suggested that the vegetal protein induces modifications in the cholesterol metabolism by the action of bioactive peptides, or by the differences in the amino acid pattern [28]. Furthermore, dietary fiber is known to cause a reduction in the intestinal absorption of cholesterol and/or bile acids, and it also decreases the triglyceride levels by inhibiting hepatic lipogenesis [29]. Some bioactive antioxidants, such as phenols, have cardio-protective actions by decreasing oxidized LDL particles [30], in addition to anti-inflammatory and anti-thrombotic effects [31].

Previous works coincide with our results regarding the cholesterol-lowering properties of amaranth. Czerwiński et al. [9] showed that amaranth flour positively influences the plasma lipid profile in rats fed with hypercholesterolemic diets, and they also proposed that this effect is related to the compounds with biological activity and the antioxidant activity in the whole seed. Escudero et al. [6] showed that the seed protein concentrate is efficient in reducing cholesterol and triglycerides levels in plasma of rats fed with hypercholesterolemic diets. Mendonça et al. [11] proposed that the bioactive peptides, present in the amaranth seed can affect the cholesterol absorption in the intestine or it can even have a direct effect on cholesterol synthesis and/or LDL uptake in liver, resulting in a cholesterol decrease in hamster's plasma. Shin et al. [32]

Table 1 Levels of lipids in serum

	CC	EC	C*Ah*	E*Ah*
Total-C (g/l)	67.67±2.58	67.60±2.30	54.8±3.11•••	53.90±5.13°°°
HDL-C (g/l)	25.00±2.41	23.00±2.91	22.67±2.25	20.60±3.78
(LDL + VLDL)-C (g/l)	40.67±2.32	44.60±1.84	32.13±3.25•••	33.30±2.90°°°
HDL-C/colesterol total	0.40	0.34	0.41	0.42

CC control casein; *EC* ethanol casein; *CAh* control *Ah; Ah* ethanol *Ah*; *Total-C* total cholesterol; *HDL-C* HDL-cholesterol; *LDL + VLDL-C* LDL + VLDL-cholesterol

Different symbols indicate statistical significance: E*Ah vs* EC→° : °°° $p<0.001$; C*Ah vs* CC→• : ••• $p<0.001$. Values are means ± SD ($n=6$)

Table 2 Levels of lipids in liver tissue

(μg/g tissue)	CC	EC	C*Ah*	E*Ah*
Total cholesterol	2755.98±269.36	3672.93±239.39*	2940.75±327.84	3170.51±368.46
Free cholesterol	1018.75±79.94	1328.80±77.54*	1132.02±57.59	1141.20±62.15°
Esterified cholesterol	1737.22±103.41	2344.13±82.09**	1808.73±112.19	2029.31±94.24°°
Triglycerides	655.23±68.66	876.10±75.34 **	728.27±70.32	774.93±63.71

CC control casein; *EC* ethanol casein; *CAh* control *Ah*; *EAh* ethanol *Ah*

Different symbols indicate statistical significance: E*Ah vs* EC→° : °$p<0.05$, °° $p<0.01$; EC *vs* CC→* : * $p<0.05$, ** $p<0.01$. Values are means ± SD ($n=6$)

published that the squalene found in amaranth can reduce cholesterol by increasing fecal elimination of steroids and interfering with cholesterol absorption in rats. Kulakoba et al. [33] showed that amaranth oil has hypocholesterolemic actions in rats. Zeashan et al. [34] revealed that ethanol extract of the whole amaranth plant, could yield a significant protection against galactosamine/lipopolysaccharide-induced hepatocellular injury, reverting the increased serum and hepatic lipids values. Despite the available information, there are no reports studying the protective effect of amaranth on alcohol intoxication.

For these reasons, we studied the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) and the LDL receptor (LDLr), which play an important role in the biosynthesis and uptake of cholesterol in the liver, respectively. Both can be regulated by the sterol regulatory element binding protein 2 (SREBP-2). On the other hand, cholesterol 7-alpha-hydroxylase (CYP7A1) is the key enzyme for cholesterol elimination through bile acids. We also measured the expression of apolipoproteine B (Apo B) that is the main protein constituent in the afferent way of cholesterol, being the most abundant protein in LDL, VLDL, IDL lipoproteins, and in chylomicrons.

Figure 1 shows the results of the expression of genes involved in cholesterol metabolism. The expression of HMG-CoAR was lower in the E*Ah* group when compared to EC ($p<0.001$) and C*Ah* ($p<0.01$), and that of LDLr was higher in E*Ah* when compared to EC and C*Ah* ($p<0.001$). HMG-CoAR mRNA levels increased in EC when compared to CC ($p<0.001$). There were no changes in the expression of SREBP-2, CYP7A1 or Apo B between the groups. From these results we infer that in the groups that received ethanol in a sub-chronic manner, amaranth had a positive effect due to the lower expression of HMG-CoAR, while the transcriptional activation of LDLr would be part of the regulatory mechanism in response to the cholesterol content, and it may also contribute to prevent the serum cholesterol increase due to ethanol.

Figure 2 shows the data of the expression of genes involved in fatty acids and triglycerides metabolism. The expression of the enzymes involved in the synthesis of triglycerides,

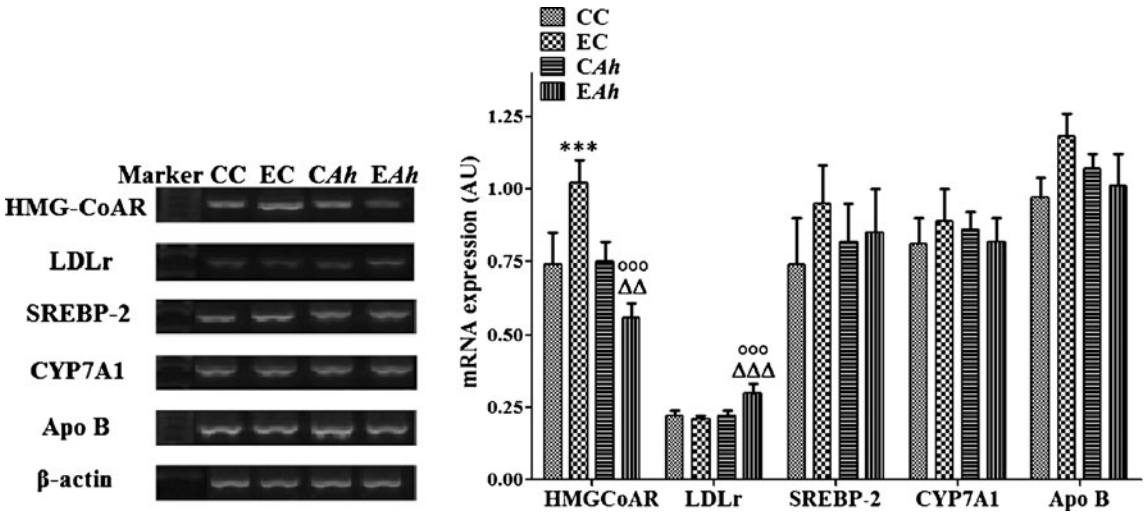

Fig. 1 RT-PCR analysis of the expression of cholesterol-related genes against β-actin as internal standard. Agarose gel images showing the PCR products and densitometric representation of the results. *CC* control casein; *EC* ethanol casein; *CAh* control *Ah*; *EAh* ethanol *Ah*; *AU* arbitrary units. *HMG-CoAR* 3-hydroxy-3-methylglutaryl coenzyme A reductasa; *LDLr* LDL receptor; *SREBP-2* sterol regulatory element binding protein 2; *CYP7A1* cholesterol 7-alpha-hydroxylase; *Apo B* apolipoprotein B. Different symbols indicate statistical significance: E*Ah vs* EC→° : °°° $p<0.001$; E*Ah vs* C*Ah* →$^{\Delta}$:$^{\Delta\Delta}$ $p<0.01$, $^{\Delta\Delta\Delta}$ $p<0.001$; EC *vs* CC→* : *** $p<0.001$. Values are means ± SD ($n=6$)

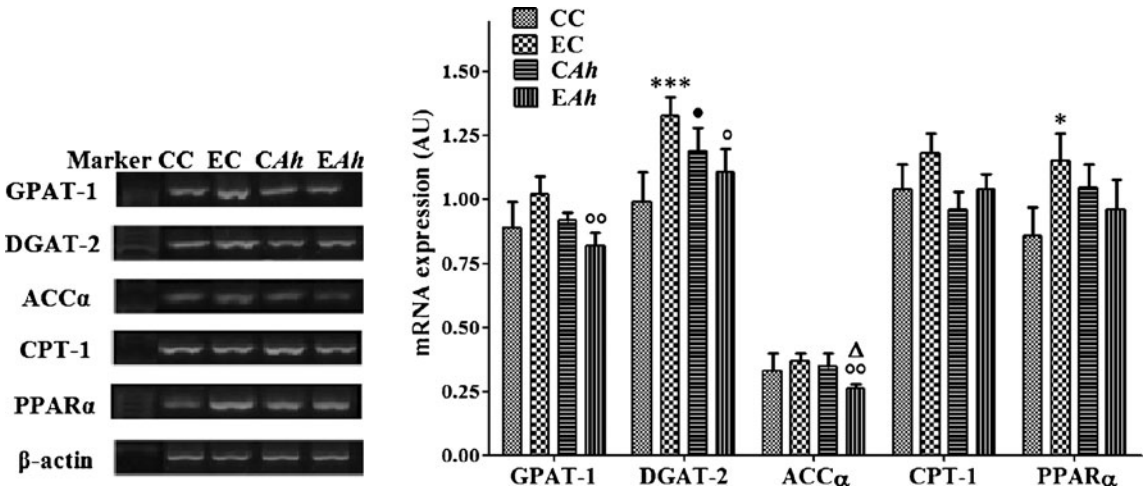

Fig. 2 RT-PCR analysis of the expression of cholesterol-related genes against β-actin as internal standard. Agarose gel images showing the PCR products and densitometric representation of the results. *CC* Control Casein; *EC* Ethanol Casein; *CAh* Control *Ah*; *EAh* Ethanol *Ah*; *AU* arbitrary units. *GPAT-1* glycerol-3-phosphate acyltransferase 1; *DGAT-2* diacylglycerol O-acyltransferase 2; *ACCα* acetyl-CoA carboxylase alpha; *CPT-1* carnitina palmitoil transferase 1; *PPARα* peroxisome proliferator-activated receptor alpha. Different symbols indicate statistical significance: E*Ah vs* EC→°: °$p<0.05$, °° $p<0.01$; C*Ah vs* CC→•: •$p<0.05$; E*Ah vs* C*Ah*→$^{\Delta}$:$^{\Delta}$ $p<0.05$; EC *vs* CC→*: *$p<0.05$, *** $p<0.001$. Values are means ± SD ($n=6$)

glycerol-3-phosphate acyltransferase 1 (GPAT-1) and diacylglycerol O-acyltransferase 2 (DGAT2) decreased in E*Ah vs*. EC ($p<0.01$) and ($p<0.05$), respectively. Considering these two genes, it is clear that in rats that were exposed to ethanol, amaranth exerts a control effect on the triglycerides synthesis; this does not happen when the protein source is casein, since an increment in DGAT-2 expression is observed when comparing EC *vs*. CC ($p<0.001$). In addition, as previously expressed, in rats treated with ethanol amaranth tends to reduce the triglycerides liver deposit; this effect would be supported by the lower expression of their synthesis enzymes. For a better understanding of these results, we determined the expression of some genes involved in the fatty acids metabolism.

We studied acetyl-CoA carboxylase alpha (ACCα), which is the regulatory enzyme in the fatty acids biosynthesis, and carnitine palmitoil transferase 1 (CPT-1), which catalyzes the entrance of fatty acids into the mitochondria and it is an important enzyme in the β-oxidation, which can be regulated by peroxisome proliferator-activated receptor alpha (PPARα).

Figure 2 also shows that the expression of ACCα decreased in E*Ah* when compared to EC ($p<0.01$) and C*Ah* ($p<0.05$). Animals fed with amaranth did not show

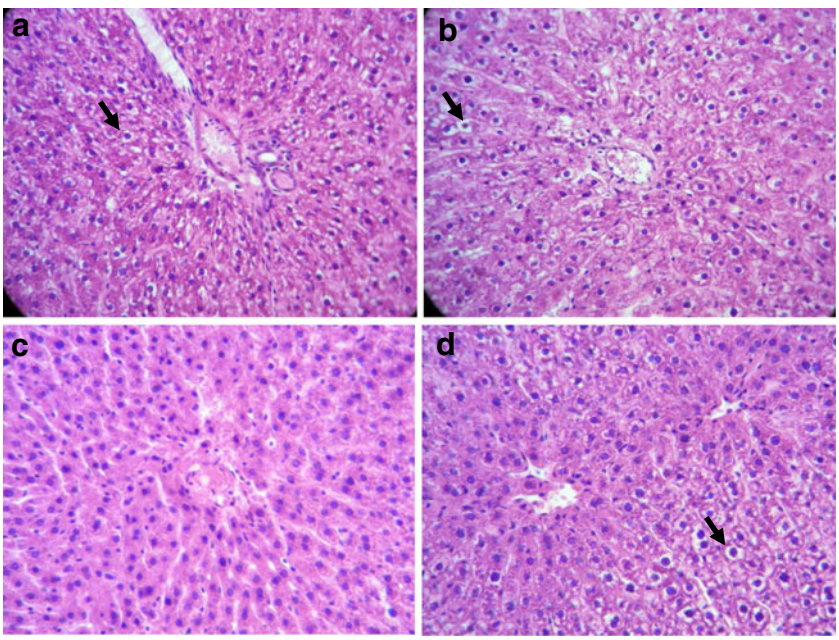

Fig. 3 Hematoxylin and eosine stained liver sections (100×). **a** CC (control casein): hepatocytes are displayed with fatty infiltrates (*arrow*). **b** EC (ethanol casein): fatty deposits are widely distributed occupying most of the cytoplasm. **c** C*Ah* (control *Ah*): the hepatocytes show a normal histoarchitecture. **d** E*Ah* (ethanol *Ah*): the fatty deposits are seen by sector

differences in the levels of CPT-1 or PPARα mRNA; instead, a significant increase in PPARα was observed in EC when compared to CC ($p < 0.05$).

It is known that ethanol can suppress the expression of PPARα, attenuating fatty acid β-oxidation and leading to an accumulation of triglycerides in the liver [35]. Amaranth could reduce the lipogenesis inductor effect of ethanol through the decrease in the expression of ACCα and the triglycerides synthesis enzymes. In addition, there would be an increased expression of PPARα in EC, when compared to CC, in response to the increased level of triglycerides in liver. This was not observed in the groups fed with amaranth, since there are no changes in the liver triglycerides content.

In general, it has been observed that increases or decreases in the expression of genes involved in the synthesis or degradation of fatty acids, cholesterol and triglycerides, where not affected by the transcription factors expression. This is due to the multiple ways of regulation, at the post-transcriptional and post-translational levels. It is important to note that in this model, the administration of ethanol is sub-chronical before the development of a more severe liver damage, so hyperlipidemia prevails and thus, we observe the first changes in liver gene expression.

Based on the results of lipids content in serum and liver, we thought it was necessary to determine if there is a simultaneous fat infiltration, since this is an important factor for the development of alcoholic hepatic disease. Therefore, we performed a hematoxilin and eosin stain.

Figure 3a and b show pictures from the CC and EC groups, respectively. Both groups exhibit fat deposits with structural modifications in the liver parenchyma. This effect is more pronounced in EC, where the fat infiltrates are widely distributed, occupying most of the cytoplasm, and have a spongy aspect. Figure 3c shows a picture of C*Ah*, where we can visualize a normal liver parenchyma, with aligned hepatocytes and conserved white spaces corresponding to the sinusoids. Figure 3d shows a picture of E*Ah,* where we can observe some lipid droplets localized by sectors. When comparing C*Ah vs.* CC (Fig. 3c and a), and in lesser extent in E*Ah vs*. EC (Fig. 3d and b), we can clearly see that amaranth decreased the fat deposit when compared to casein. These data is consistent with the biochemical and molecular results shown in this study.

Conclusion

Our findings suggest that in the presence of ethanol, the integral flour of *Amaranthus hypochondriacus* has a positive effect on lipids, which may be preventing the increase in serum cholesterol through a higher expression of the LDL receptor, as well as a decrease of liver cholesterol, due to changes in the expression of HMG-CoAR. On the other hand, amaranth seems to attenuate the tendency of ethanol to stimulate the fat deposit in liver, by decreasing the expression of ACCα and enzymes involved in triglycerides synthesis. These properties could be mainly attributed to the bioactive components in the amaranth flour, such as the proteins, fatty acid profile, soluble dietary fiber, squalene and flavonoids content.

For all these reasons, besides being recognized as a nutritive source and an antioxidant resource, amaranth should also be recommended to prevent the alterations in the lipid metabolism induced by alcohol and other harmful agents. Figure S2 shows a schematic diagram illustrating possible effects of *Amaranthus hypochondriacus* flour on the lipid metabolism, in the liver of rats sub-chronically treated with ethanol.

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Conflict of Interest The authors declare that they have no conflict of interest.

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