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EFFECTS OF PECTIN FROM CARNAUBA (*COPERNICIA PRUNIFERA*) FRUITS ON THE ACTIVITY LIVER GENE EXPRESSION OF KEY PROTEINS INVOLVED IN LIPID METABOLISM IN APOE KNOCKOUT MICE FED A HIGH-FAT DIET

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ABSTRACT

Pectin is a polysaccharide that has an important hypolipidemic potential. However, pectin isolated from carnauba fruits (*Copernicia prunifera*) has never been evaluated in *in vivo* models of dyslipidemia. Thus, we evaluated for the first time its*in vitro* and hypolipidemic effects in ApoE$^{-/-}$ mice fed a high fat diet (HFD). The treatment with pectin (150 and 300 mg/kg/day) reduced significantly of total cholesterol, LDL-C, glycemia, liver AST and lipid peroxidation, as well as induced downregulation of hepatic HMGCR transcription, a key enzyme for the synthesis cholesterol. In addition, pectin did not alter renal or hepatic functions in mice, confirmed by the absence of cytotoxicity tested by the MTT model. Thus, pectin supplementation extracted from carnauba showed hypolipidemic effects, without causing noticed adverse effects. Altogether our findings support this bioproduct with potential therapeutic usefor dyslipidemia.

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INTRODUCTION

Dyslipidemia is an epidemic health problem thatmay lead to life-threatening cardiovascular diseases (CVD), the most common cause of death, with around 17.5 million deaths worldwide annually, predominantly in lower income countries (World Health Organization, 2017a). This condition is mainly due to the increase in blood total cholesterol, a well-known atherogenic factor, as well as low density lipoprotein (LDL-C) and triglycerides fractions, conversely with the reduction of the antiatherogenic high density lipoprotein (HDL) levels (Stone *et al.,* 2014). A great number of cholesterol- lowering drugs have been in the market with overall good efficacy against dyslipidemia. However, such conventional synthetic drugs are not free from side effects, and may potentially cause rhabdomyolysis, myopathy, and liver enzyme abnormalities (Lotteau *et al.,* 2016). Thus, the demand for natural products, including compounds from plants has grown, bringing newhopes to dyslipidemic populations, towards more positive and safe results(Thaipitakwong and Aramwit, 2017). Breakthrough discoveries in this area are encouraged every day, many of them evolving from the popular knowledge, experienced over many years, and then reaching the pharmaceutical industry (Gonçalves and Pasa, 2015). Thus, biomes like the *caatinga*, a native semi-arid vegetation of the Brazilian Northeast region, have boasted in biodiversity and economic potential (Silva and Cechinel Filho, 2012), with several species of plants still unexplored by researchers and industry, but well empirically diffused by the communities with numerous domestic applications(Cartaxo *et al.,* 2010). Recently,

research has pointed out the therapeutic effect of novel substances extracted from a *caatinga* native palm tree, named *carnauba* (*Copernicia prunifera*), which has shown biotechnological potential (Arruda Filho *et al.,* 2017; Paim *et al.,* 2017; Rodrigues *et al.,* 2014). Carnauba belongs to the family Arecaceae, native to the Brazilian Northeast region, but also present in the central region of the country, as well as in other countries of South America,Asia and Equatorial Africa. It is also known as the "tree of life", because everything is harnessed from this tree for domestic and industrial processing (Crespo, 2007). Carnauba leaf wax is the product with the highest economic value, while its fruits are little explored. Basically, they are used for consumption by livestock and to the production of liqueurs, jams, jellies, biscuits among others from their pulp (Carvalho, 2005; Crespo, 2007). Rufino *et al.*, (2009) in their work evaluating the quality for *in natura* consumption of tropical fruits, found that carnauba fruits have 1.08% of pectin when ripe.

Pectins belong to a group of soluble fibers, displaying complex branched polysaccharides, including α-1,4-D-galacturonic acids that under partial methyl esterification, and harboring several neutral sugars residing in their side chain, such as L-rhamnose, L-arabinose and D-galactose (Mohnen, 2008). In addition, other polysaccharides consisting of arabinose and/or galactose associated with pectic polysaccharides, such as arabinogalactan (AG) (type I and type II) have already been isolated. AG-II can be combined with proteins, named arabino galactan proteins (AGPs)(Sriamornsak, 2003). Pectins, when ingested, can increase satiety, and thuslower the energy intake, additionallythey bind to cholesterol and bile acids, favoring the endogenous cholesterol clearance through LDL-c. Furthermore, pectins may be fermented by intestinal bacteria producing short chain fatty acids (SCFAs), that promote inhibition of hepatic cholesterol synthesis and/or cholesterol redistribution from plasma to liver (Richards *et al.*, 2016). In view of the pectin potential benefits for the treatment of dyslipidemia, the aim of this study was to verify whether the carnauba fruit-extracted pectin could lower blood cholesterol levelsfromchronically high-fat diet-fedapoE knockout mice.ApoE knock-out ($^{-/-}$) mice are well-known to spontaneously develop dyslipidemia, andits onset is rapidly accelerated by the intake of high-fat diets.As far as we know, no studies have explored the carnauba-derived pectin on lipid metabolism in animal models.

MATERIALS AND METHODS

Plant Material, Isolation and Purification of Pectin from C. prunifera: The green fruits of *Copernicia prunifera* (Mill.) H. E. Moore were collected in Aracati, Ceará (northeastern Brazil), and used for extraction. Other chemicals were purchased from Sigma (St. Louis, MO). The green fruit pulps were separated from 100g of the sample. The extraction of pectin was carried out by applying 0.25% ammonium oxalate solution (pH 4.6) for 1 hour at 80°C, followed by a solid: 20:1 liquid extraction. The extract was then filtered. The filtrates were combined, and the pH was adjusted to 6 with a 0.1 N sodium hydroxide solution; the sample was then concentrated to a volume of 100 mL (10: 1). Of these, 300 mL of 95% ethanol were added to the concentrate to precipitate the pectin. The pectin was then filtered with a Buchner funnel. The filter residue was dissolved in 100 mL of water and distilled through a pad of celite. This was repeated until a clear liquid was obtained and then later lyophilized to yield 2.3% (Paim et al., 2017).

Cytotoxicity assay: Kidney epithelial cells from African green monkey *Chlorocebussabaeus*(VERO cells; kindly provided by the Research Laboratory on Pathogenic Bioagents of the University of Fortaleza, Brazil) were seeded in 96-well plates at the concentration of $2x10^5$ cells/mL and cultured for 24 hours at 37 °C in L-15 (Cultilab, Bazil) medium with 2% fetal bovine serum (Cultilab, Bazil) and 1% penicillin/streptomycin (Sigma, USA). After 24 hours, the medium was removed, and the carnauba-derived pectin added in triplicate with serial dilutionsat an initial concentration of 2 mg/mL. The pectin cytotoxicity test was evaluated by the MTT method (3-(4,5-dimethylthiazol-2yl) -2,5-diphenyltetrazolium bromide). The MTT assayrelyies on the conversion of the water soluble MTT to an insoluble formazan. After 72 hours of incubation at 37 °C, samples were discarded and 50 µL of MTT (5 mg/mL) were added to the wells. The plate was incubated for 4 hours at 37°C under the light. Afterwards, MTT solution was removed and 50 µl of DMSO were added with slight stirring for the full dilution of the crystals. The solubilized product was analyzed in a spectrophotometer at 540 nm wavelength.

Animals: A total of 50 male $apoE^{-/-}$ mice, weighing between 25 and 30g, kindly provided by the University of Fortaleza, Ceará, Brazil, were used in the study. All animals were kept in a 12 hours light/12 hours dark cycle with free access to water and standard rodent diet until the onset of the experiment. Experimental mice were weighed on days 30 and 60 following the diets. The animals were acclimatized for 1 week prior to the experiment at the State University of Ceara vivarium. All the experiments in the study complied with the ethical principles of animal experimentation guidelines from the Ethics Committee for Animal Care of the State University of Ceara.The approved protocol is registered under the protocol number # 42582992/2016.

Induction of dyslipidemia by chronically givenhigh-fat diet: $ApoE^{-/-}$ mice were fed a high fat diet (HFD) for 30 days, consisting of parmesan cheese (10%), cholesterol (1%) and cholic acid (0.1%). Ingredients such as corn flour and soybean, which are protective for cardiovascular diseases, were replaced with refined wheat flour. In addition, a mixture of milk chocolate (Nescau®) (10%), whole milk powder Ninho® (10%) and water (80%) were available *ad libitum*to the animals (with the exception of the negative control group) for a period of 4 hours. The full composition of the diet is described in Table 1. After 4h, blood samples were harvested for confirmation of dyslipidemia. Experimental mice continued to receive the high-fat diet and were divided into five groups (n = 7) that received the following treatments for two months:

- Standard diet group (SD) was fed with standard rodent diet and receiving drinking water;
- High Fat Diet group (HFD) was fed an enriched diet and receiving drinking water;
- Simvastatin group (SIMV) was fed an enriched diet and received a dose of 20 mg/kg/day ofsimv.
- Groups that received pectin (PEC150 and PEC300) were fed the enriched diet and received doses of 150 and 300 mg/kg/day of pectin, respectively.

Collection and analysis of serum lipids: At the end of each month of treatment, experimental mice were fasted for 8 hours for serum lipid analyses. Blood samples were collected from the retro-orbital plexus, using capillary tubes at 30 and 60-day treatment. The serum was obtained fromclotted blood at room temperature with centrifugation at 600 x g for 10 minutes. The obtained serum was stored at -20°C for the determination of biochemical markers. Serum samples were assessed for triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and urea using the Metrolab 23300 kit 1.7. following the manufacturer's specifications. LDL-C levelswere calculated by the following equation: LDL-C = CT-HDL-TG/5 (Friedewald et al., 1972).

RNA extraction and quantitative real time PCR (qPCR): Liver samples were cut into small fragments, immersed in RNAlaterandfrozen at -20°C until analysis. For each sample, 2 µg of total RNA were used for cDNA synthesis using the high capacity cDNA ™ RNA kit (Applied Biosystems®). As a control, the RNA pool from animals from each group was used for the reaction without reverse transcriptase. Analysis of mRNA expression of genes related to lipid metabolism, such as ApoAI, LCAT, HMG CoA reductase (HMGCR), and LDLr, as well as TNF and IL-4 cytokines, were analyzed by RT-PCR with intercalating dye and normalized with

B2M and RPLP0 reference genes. The primers used are listed in Table 2. The qPCR reaction was performed in triplicate using: 10 µl GoTaq® qPCR Master Mix (Promega®), 10 pmol of each primer and cDNA amount equivalent to 20 ng of total RNA extracted, totaling 20 µL per reaction. The qPCR was performed in real-time thermal cycler (Esco Swift, Esco) with the following conditions: 10 min at 94 °C (1 cycle); 20 sec to 94°C, 20 sec to 60°C and 20 sec to 72°C, with acquisition at the end of this last step (40 cycles); and two min at 72°C (1 cycle). At the end of the PCR step, the amplified products were analyzed by melting curve. For each primer, controls without template and without reverse transcriptase were also tested in triplicate. From the mean values Cq of each group, the relative expression of each gene was calculated by the comparative method Cq (ΔΔCq), using as reference the geometric mean of the constitutive genes for the normalization of the expression results.

Evaluation of Liver Oxidative Stress

Nitrite Quantification: The concentration of nitrite was determined according to a pre-established method (Green et al., 1982). For this experiment, 100 µl of the Griess reagent (1% sulfanilamide/1%N-(1-naphthyl) ethylenediamine hydrochloride/ 1% H3PO4/ distilled water in the ratio of 1:1:1:1) was added to 100 µl of the hepatic homogenate (diluted in potassium phosphate buffer) and incubated at room temperature for 10 min. The standard curve was elaborated with various concentrations of $NaNO_2$ (ranging from 0.75 to 100 µM) under the same conditions. The blanks were prepared by the addition of 200 µL Griess reagent, 200 µL phosphoric acid and 200 µL water. The absorbance was measured by a microplate reader at 560 nm.

Quantification of Malondialdehyde (MDA): The quantification of malondialdehyde was determined as described elsewhere (Draper and Hadley, 1990). 250µl of the hepatic homogenate in PBS buffer (10%) were incubated at 37°C for 60 min; then, 400µL perchloric acid (35%) were added and centrifuged at 3000 x g for 10 min at 4 ° C. Subsequently, 200 µL of thiobarbituric acid (TBARS 0.8%) was added and incubated at 95-100°C for 30 min. The standard curve was elaborated with various concentrations of TBARS in potassium phosphate buffer (ranging from 0.78 to 100 µM) under the same conditions. Blanks were prepared by the addition of TBARS in potassium phosphate buffer and the absorbance was measured withthe aid of a microplate reader at 532 nm.

Statistical analysis: Data were expressed as mean ± standard error of the mean (SEM). The significance of the differences between the groups were evaluated by analysis of variance (ANOVA), followed by the *Tukey* test. A value of $p<0.05$ was considered significant.

RESULTS

Cytotoxicity of pectin: Carnauba fruit-derived pectin had no cytotoxic effect against *Vero* cells (IC50 > 2 mg/mL), up to 72 hours of incubation, as assessed by the MTT assay.

Effect of carnauba fruit-derived pectin on the biochemical profile from hypercholesterolemic mice and controls: In order to assess whether carnauba-derived pectin could improve dyslipidemia in apoE knockout mice challenged with HFD, we analyzed serum levels of TC, TGL, HDL-C and LDL-C. After 60 days of treatment, a significant reduction in TC and LDL-C levels was observed for PEC150 (769.3±30.7 and 580.0±34.6 mg/dL) and PEC 300 (827.0 ±2.2 and 637.6±2.3 mg/dL), both $p < 0.001$, when compared to the untreated-HFD group (1266.0±21.5 and 1150.0±26.9 mg/dL), a marked reduction above 30%. This becomes important, since the high fat diet was responsible for doubling these values as opposed to the controls receiving the standard chow diet (Table 3). The experimental high-fat diet was not able to raise triglyceride levels, and the pectin-treatmentdid not alter these high values until the end of the experiment. In addition, $ApoE^{-/-}$ mice receiving HFD showed increased serum HDL-C levels. SIMV and PEC300 treatments failed to alter these values.

Effect of pectin on renal and hepatic toxicity parameters fromhypercholesterolemic mice and controls: With respect to liver transaminase analysis, a sensitive indicator of liver function alterations, the HFD increased AST levels (4.26 times $p < 0.001$) compared to the SD group, suggestingeither liver dysfunctionor toxicity (Shang et al., 2014).Treatment with pectin abrogated this increase ($p<0.001$), showing values of 48.4±4.6 and 50.8±1.8 (U/L) for PEC 150 and PEC 300, respectively, against 128.8±9.2 (U/L) of the HFD group with more than 2-fold decrease. Regarding renal metabolites, urea levels did not show changes between groups. However, creatinine was markedly increased in the group receiving simvastatin compared to untreated HFD group (Table 3).

***Effect of carnauba fruit-derived pectin on food intake, body weight and relative liver weight of* HFD-fed mice:** Chronically given HFD increased body weight gain from untreated mice, compared to the standard chow diet (SD) group. Of note, both pectin treatments (PEC150 and PEC300) attenuated this increase at 60 days of the HFD, despite the increase in chocolate milk intake at 30 days.HFD was not able to significantlyincrease relative liver weight, effect which was not modified by all treatments (Table 4).

Effect of carnauba fruit-derived pectin on the hepatic mRNA levels of ApoAI, LCAT, HMGCR, LDLr, LXRα, TNFα, and IL-4: Chronically given HFD reduced the liver mRNA levels of ApoAI (Fig. 1A), LXRα (Fig. 1B), and LDLr (Fig. 1C) in apoE ($^{-/-}$), regardless of PEC treatment. LDLr was drastically elevated by simvastatin treatment. Out of the five genes involved in lipid metabolism, only HMGCR (Fig. 1D) showed reduction (~ 4x) of its expression following pectin treatment at 150 and 300 mg/kg/day,($p<0.0001$ each), compared to the HFD group, whereas simvastatin could not affect that after 60 days of treatment (Fig. 1A). However, pectin induced downregulation of LCAT ($p<0.01$) compared to the HFD group (Fig. 1E). HFD and pectin treatment were not able to alter TNF (Fig. 1F) transcription levels. However, the diet used in the study increased significantly the IL-4 (Fig. 1G) mRNA concentration in the hepatic tissues of the animals, a result that was accompanied by the PEC300 group and not by the group receiving simvastatin (Figure 1).

Effect of carnauba fruit-derived pectin on oxidative stress in ApoE$^{-/-}$ mice fed a HFD: The synthesis and accumulation of liver MDA (malondialdehyde), a marker of cell membrane peroxidation (and oxidative stress), was significantly lower in pectin treated animals at doses of 150 mg/kg (18.2%) and 300 mg/kg (17.2%) when compared to the standard drug simvastatin (Figure 2A). However, nitrite levels did not show changes after treatment (Figure 2B). Nitrite levels, also related with oxidative stress, were significantly lower in pectin treated animals at doses of 150 mg/kg (47.8%), 300 mg/kg (50.9%) and simvastatin (47.7%) when compared to the HFD group (Figure 2A). HFD reduced liver levels of TBARS. Interestingly, simvastatin treatment significantly increased TBARS levels, whereas pectin treatment did not, comparedto the SD group (Figure 2B).

DISCUSSION

The Western diet, enriched with fat and sugar, has undoubtedly contributed to the epidemic increase in cardiovascular events, mostly due tothe rise of dyslipidemia worldwide, especially in genetically prone individuals. Thus, a great deal of attention has been given to loweringabnormal blood levels of total cholesterol (TC) and low-density lipoproteins (LDL-C),promptingthe pharmaceutical industry and researchers to pursue novel safe therapies against dyslipidemia. Investigations in the field of natural products have shown therapeutic potential of chemical compounds extracted from various parts of *Copernicia prunifera* (Miller) – carnauba plant. Recent studies from our group have pointed out to positive results of these substances in

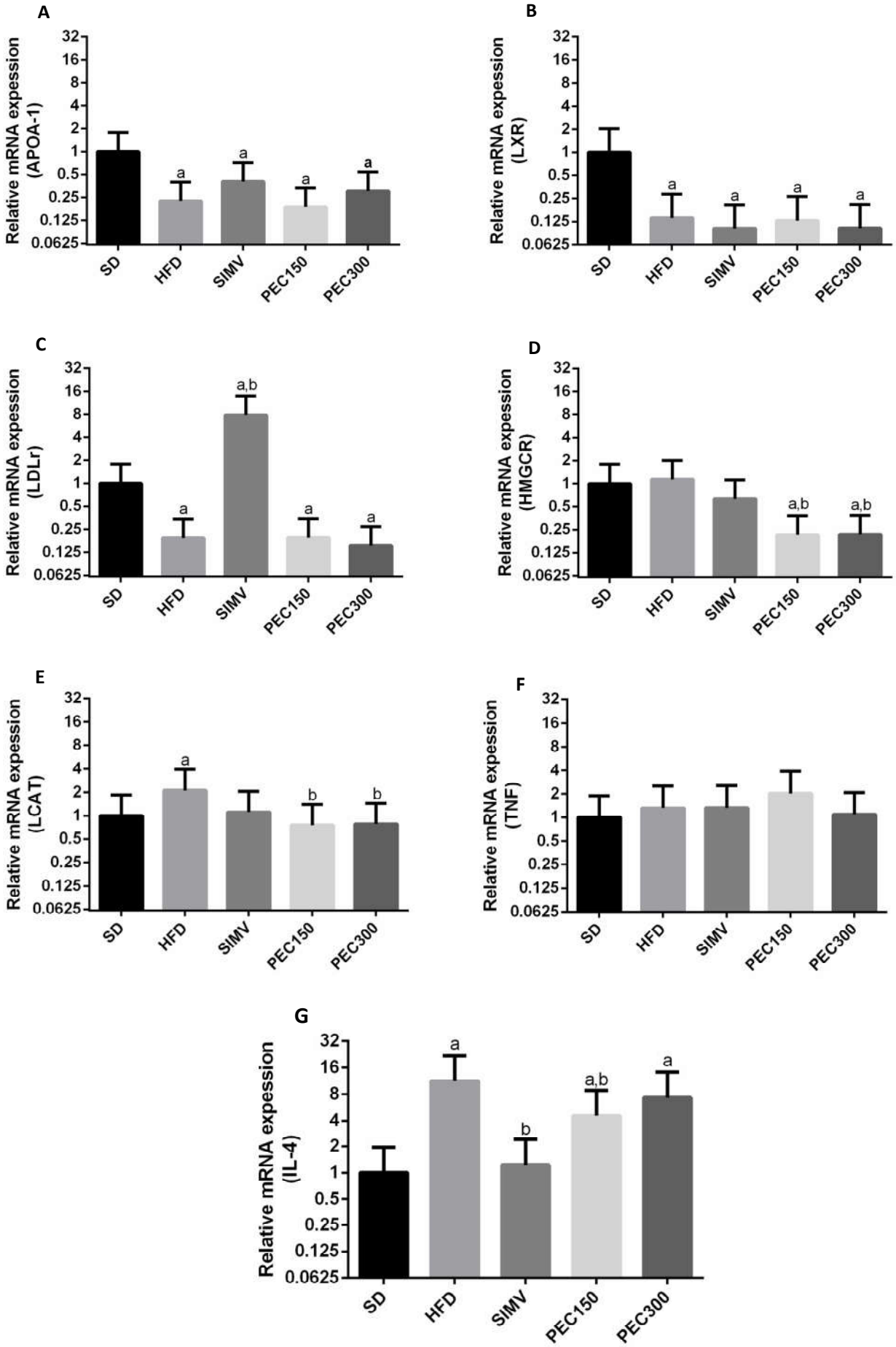

Figure 1. Effects of carnauba fruit-derived pectin and simvastatin on relative expression of mRNAs from genes involved in lipid metabolism (A-F) and inflammation (G) from ApoE$^{-/-}$ mice receiving high-fat diet. SD=standard diet; HFD= high fat diet; SIMV= HFD + simvastatin (20 mg/Kg), PEC 150 and PEC 300= HFD + pectin at doses of 150 and 300 mg/Kg/day, respectively; a= p<0.05 vs SD; b = p<0.05 vs untreated HFD (ANOVA and *Tukey* test).

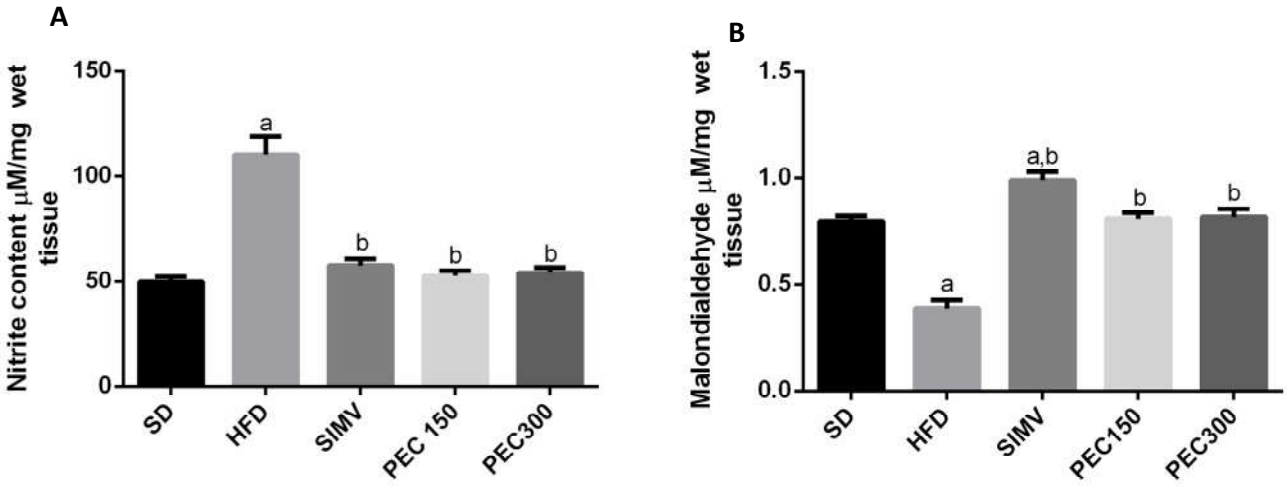

Figure 2. Effects of carnauba fruit-derived pectin and simvastatin (20 mg/Kg) on malondialdehyde (MDA) and nitrite levels in the hepatic tissue of ApoE$^{-/-}$ mice fed a high-fat diet and controls receiving a standard diet (SD). SIMV= HFD + simvastatin; PEC 150 and PEC 300, carnauba-derived pectin at dose of 150 and 300 mg/kg/day. a=p <0.05 vs SD (ANOVA and *Tukey* test).

Table 1. Composition of experimental high-fat diet associated with milk chocolate

Ingredients	**%**
Wheatflour	61.1
Sucrose	9.0
Casein	4.0
Butter	10.0
Gratedparmesancheese	10.0
L-cystine	0.3
Cholesterol	1.0
Colicacid	0.1
Mix Vitamins	1.0
Mix Minerals	3.5
TOTAL	100
Macronutrient composition of HFD associated with milk chocolate	
Carbohydrate	48.1%
Protein	10.9%
Lipid	41.0%

Table 2. Primers Used for Real-Time PCR Analysis

Gene		**Primer**	**Sequence (5`-3`)**		
ApoAI		Forward	TCAAAGACAGCGGCAGAGAC		
ApoAI		Reverse	CACCTTCTGGCGGTAGAGCTC		
LCAT		Forward	GTAACCACACACGGCCTGTCAT		
LCAT		Reverse	GTTGAAATCCAGCCAGATGGT		
HMGCR		Forward	CCGGCAACAACAAGATCTGTG		
HMGCR		Reverse	ATGTACAGGATGGCGATGCA		
LDLr		Forward	GAGGAACTGGCGGCTGAA		
LDLr		Reverse	GTGCTGGATGGGGAGGTCT		
LXR – α		Forward	TCAGCATCTTCTCTGCAGACCGG		
LXR – α		Reverse	CATTAGCATCCGTGGGAACA		
TNF		Forward	AGGGATGAGAAGTTCCCAAATG		
TNF		Reverse	CACTTGGTGGTTTGCTACGAC		
IL-4		Forward	ACAGGAGAAGGGACGCCAT		
IL-4		Reverse	GAAGCCCTACAGACGAGCTCA		
RPLP0		Forward	GCTTCATTGTGGGAGCAGACA		
RPLP0		Reverse	CATGGTGTTCTTGCCCATCAG		
B2M		Forward	CATGGCTCGCTCGGTGACC		
B2M		Reverse	AATGTGAGGCGGGTGGAACTG		
ALT	27.3±3.9	21.1±3.2	34.8±4.5	36.4±6.7	33.2±5.0
URE	66.1±2.3	67.4±7.6	57.8±4.8	50.6±2.5	48.9±3.3
CREAT	0.7±0.2	0.8±0.2	2.6±0.4^{a,b}	0.6±0.1	0.49±0.1

Table 3. Effect of carnauba fruit-derived pectin on diet-induced dyslipidemia in apoE knockout mice after 60 days of treatment

Parameters	SD	HFD	HFD+SIMV	HFD+PEC150	HFD+PEC300
TC	536.4±39.3	1266.0±21.5[a]	1263.0±27.2[a]	769.3±30.7[a,b]	827.0±22.2[a,b]
TGL	293.0±14.7	304.9±10.5	409.9±43.8[a,b]	754.9±12.2[a,b]	769.8±9.8[a,b]
HDL-C	10.4±1.7	55.1±9.4[a]	16.6±4.1[b]	25.3±4.7[b]	38.9±9.1[a]
LDL-C	484.5±42.7	1150.0±26.9[a]	1169.0±35.7[a]	580.0±34.6[b]	637.6±22.3[a,b]
AST	30.3±3.1	128.8±9.2[a]	94.2±9.8[a,b]	48.4±4.6[b]	50.8±1.8[b]
ALT	27.3±3.9	21.1±3.2	34.8±4.5	36.4±6.7	33.2±5.0
URE	66.1±2.3	67.4±7.6	57.8±4.8	50.6±2.5	48.9±3.3
CREAT	0.7±0.2	0.8±0.2	2.6±0.4[a,b]	0.6±0.1	0.49±0.1

SD=standard diet; HFD= high fat diet; SIMV= simvastatin (20 mg/Kg), PEC 150 and PEC 300= pectin at doses of 150 and 300 mg/Kg/day, respectively; TC= total cholesterol (mg/dL); TGL= triacylglycerol (mg/dL); HDL-C= high density lipoprotein (mg/dL); LDL-C= low density lipoproteins (mg/dL); AST= aspartate aminotransferase (U/L); ALT= alanine aminotransferase (U/L); CREAT= creatinine (mg/dL); URE= urea (mg/dL). a= p<0.05 vs SD; b = p<0.05 vs untreated HFD (ANOVA and *Tukey* test).

Table 4. Effect of carnauba-derived pectin on body weight, *relative liver weight,* food and water intake of HFD-fed mice.

Parameter	Time (days)	SD	HFD	HFD+SIMV	HFD+PEC150	HFD+PEC300
FI (g)	30	4.0±0.2	2.0±0.1[a]	1.8±0.2[a]	2.6±0.2[a]	2.4±0.1[a]
	60	3.6±0.3	1.9±0.31[a]	1.5±0.23[a]	2.9±0.2[b]	2.4±0.2[a]
CI (mL)	30	*-	26.5±3.8	33.5±4.4[b]	35.3±3.4[b]	35.6±2.7[b]
	60	*-	48.5±4.4	44.5±5.3	49.1±1.2	45.4±1.9
WC (mL)	30	30.9±6.9	20.0±5.8	25.0±8.7	28.3±6.0	29.3±1.9
	60	51.3±10.9	21.8±5.2	15.0±3.1	28.3±1.4	38.6±1.5
WG (%)	30	98.3±1.1	111.4±1.5[a]	101.8±1.3	102.6±2.2	104.9±3.1
	60	100.1±2.3	131.5±3.9[a]	94.27±5.2[b]	110.4±5.6[b]	112.2±6.6[b]
RLW	60	4.0±0.1	5.5±0.2	5.5±0.5	5.5±0.5	5.2±0.4

FI= food intake; CI= chocolate milk intake; WC= water consumption; WG=weight gain (% of the initial weight – day 0). RLW=relative liver weight; SD=standard diet; HFD= high fat diet; SIMV= simvastatin (20 mg/Kg), PEC 150 and PEC 300= pectin at doses of 150 and 300 mg/Kg/day, respectively; *SD did not receive chocolate milk. a= p<0.05 vs SD; b = p<0.05 vs untreated HFD (ANOVA and *Tukey test*).

experimentally induced diabetes (Rodrigues *et al.,* 2014) and dyslipidemia (Arruda Filho *et al.,* 2017; Guedes *et al.,* 2010; Paim *et al*., 2017), besides their use in food industry (de Freitas *et al.,* 2019). The pectin from thecarnauba fruit pulp was first described by Rufino *et al.*, (2009). However, this work is the first to report the effects of carnauba-derived pectin in in vitro toxicity assays, and the effects on liver lipid metabolism, liver inflammation gene expression and oxidative stress using the apoE knockout mice. In our study, the Carnauba fruit-derived pectin, a polymer mainly of poly-α-(1→ 4) -D-galacturonic acid (Paim et al., 2017), did not induce toxicity to Vero cells up to 72 hours of incubation. Our result agrees with other literature reports documenting no cytotoxic activity of other types of pectin in healthy cell lines(Almeida et al., 2015; Leclere et al., 2016). Of note, pectin is a constituent of all terrestrial plants and is considered harmless from a toxicological point of view according to JECFA (Joint FAO/WHO Expert Committee on Food Additives) (World Health Organization, 2017b).

In earlier studies, we have addressed the effects of the aqueous extract of carnauba fruits in Swiss mice challenged by a high-fat diet (unpublished data). The aqueous extract (150 mg/kg/day) reduced serum cholesterol and triglyceride levels, as well as significantly increased HDL-C levels after 90 days of treatment, without causing hepatic and renal damage, protective effects that may have been attributed to the pectin content. As expected HFD induced the increase of TC and LDL-c in apoE deficient mice. This effect was markedly reversed when these animals received carnauba-derived pectin at doses of 150 and 300 mg/kg/day, supporting a protective role in dyslipidemia. Previous studies have shown that pectin plays an important role in the process of inhibiting lipid absorption, or also integrates into the synthesis of cholesterol from volatile fatty acids. Among the most reported mechanisms that explain the reduction of fiber-mediated blood cholesterol is the biliary excretion pathway (bile acids and steroids) leading to the control of bile acid biosynthesis (Zhu *et al.*, 2013a, 2017, 2015). The HFD challenge significantly altered the liver enzyme AST, suggesting tissue injury. The pectin treatment prevents the increase of AST (Samout et al., 2016a), and thus, positively impacted liver function. AST and ALT hepatic enzymes have been reported to increase in dyslipidemia as a result of elevated dietary cholesterol content, which breaks down cell membranes with enzyme leakage into the bloodstream (Shang et al., 2014), or yet, this extravasation may be due to damage to skeletal myofibrils, resulting in systemic exposure with risk of dose-related muscular toxicity (Bodié et al., 2016). In addition, creatinine, a biomarker of kidney function, was significantly increased in the group receiving simvastatin, but not following pectin treatment(Wilkinson et al., 2014), suggesting less side effects than this conventional lowering-cholesterol drug. In particular, HFD significantly increasedliver weight of the experimentalapoE null mice. Liver enlargement (hepatomegaly) can be explained in part by the accumulation of triglycerides resulting from the absorption of dietary fatty acids and may increase liver response to secondary insults such as oxidative stress (Paim *et a*l., 2017). A hyperlipidic diet, especially in ApoE deficient mice, may trigger the elevation of oxidative stress and inflammation (Stamenkovic et al., 2019). In addition, endothelial modifications may lead to other effects, such as the intensification of platelet aggregation, proliferation of smooth muscle cells, adhesion of monocytes to the endothelium and increased nitrite levels (Brant *et al.,* 2014; Samout *et al.,* 2016b).

Carnauba fruit pectin reduced nitrite levels, an indirect marker of nitric oxide production a parameter of hepatic oxidation, compared to the HFD (Barroso et al., 2019). On the other hand, the levels of MDA were reduced with HFD, whereas the treatments (pectin and simvastatin) had values similar to the SD group. The reduction of lipid peroxidation in HFD group, induced by reactive oxygen species (ROS) oxidant effect, impacts local inflammatory reactions, through a compensation mechanism. According to Li *et al.,* (2018), HF-fed mice that orally ingested pectin for eight weeks exhibited improvements in lipid metabolism, and decreased oxidative stress and inflammation through a mechanism regulated by the mitogen-activated protein kinase pathway. High antioxidant activity *in vitro* and *in vivo* had already been reported for pectins previously (REF), that support our findings of lower lipid peroxidation process seen with carnauba-derived pectin. Penta-oligogalacturonide (HPPS) of haw pectin (*Crataegus pinnatifida*Bge) exhibited concentration-dependent effects against superoxide anions, hydroxyl and DPPH radicals, significantly increasing the activity of superoxide dismutase, catalase, glutathione peroxidase, total antioxidant capacity and levels of glutathione, with reductions of malondialdehyde content in the liver of mice fed a high-fat diet (Li *et al.*, 2014). Among the groups that received HFD, no significant increase in hepatic levels ofTNF-α mRNA was found.

Nonetheless, HFD was seen to elevate liver IL-4 mRNA transcription, suggesting that the rise of liver IL-4 activity study may be due to a compensatory effect to assist in tissue repair resulting from fat-rich diets in the liver *(An et al.,* 2019). In our work, the effect of HFD on stimulating IL-4 production may counterbalance tissue injurywith the aid of immunological modulation via T helper cell type 2 (Th2), including the production of more IL-4 andIL-13, which can suppress inflammatory responses (Zhou *et al.,* 2017). Considering that carnauba fruit-derived pectin is safe and based on its effects on serum lipid levels, we hypothesized that pectin could also improve reverse transport of cholesterol. Compared to HFD, PEC groups (150 and 300 mg/Kg) showed downregulation of liver 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) gene expression, whereas simvastatin did not alter thisparameter following 60 days of treatment. The decrease in the amount of HMGCR mRNA transcripts is considered relevant because HMGCR is a key enzyme in the endogenous production of cholesterol, an important therapeutic target for dyslipidemia treatment. It is worth mentioning that our data support what Zhu et al., (2013) found, after using a pentassaccharide pectin of *Crataegus pinnatifida* Bunge. Var. major in *kunming* mice fed with HFD. Pectin metabolites after bacterial fermentation can give rise to short chain fatty acids - SCFA (acetate, butyrate and propionate), which cross the bloodstream and may interfere with hepatic metabolism, altering the gluconeogenesis and lipogenesis (Richards *et al.,* 2016).

Acetate is a substrate for the synthesis of endogenous cholesterol, via acetyl-CoA, which affects plasma cholesterol levels (Hara et al., 1999), whereas propionate has a recognized role in reducing de novo synthesis of cholesterol through inhibition of (HMGCR) (Besten *et al*., 2013; Escudero and Gonzalez, 2006), as well as stimulating lipoprotein secretion containing ApoA-IV, and regulating reverse transport of cholesterol (Marcil *et al.,* 2003; Nazih *et al.,* 2001). Thus, microbiota dependent SCFA can play a key role in modifying lipid metabolism and thus may in part explain the beneficial effects of carnauba fruit pectin in improving liver total cholesterol. On the other hand, pectin did not have the expected effect of increasing liver LDLrtranscription. LDLr transcription is tightly regulated by changes in concentrations and demand for intracellular cholesterol through a feedback response, which partly explains the reduction of its expression in the hepatic tissues of hypercholesterolemic mice (Wijers *et al.,* 2019). The HFD induced downregulation of the LXR, ApoAI mRNA, LDLr when compared to the SD group. Excessive liver cholesterol is lipogenic through the activation of the liver X receptor (LXR), besides being an important mechanism that also acts on the biosynthetic route of cholesterol (Jia et al., 2010), potencial explanation for the cholesterol-lowering properties of carnauba fruit-derived pectin. LCAT (Lecithin cholesterol acyltransferase) is an enzyme produced and secreted by the liver, involved in the reverse transport of cholesterol, catalyzing the esterification of free cholesterol in HDL particles (Rosenson et al., 2012).

This process is defined by the conversion of cholesterol and phosphatidylcholine to cholesterol ester and lysophosphatidylcholine being a critical process for HDL maturation (Czarnecka and Yokoyama, 1996). The role of LCAT in atherosclerosis remains controversial in view of transcriptional changes in the LCAT gene locus, presence of cholesteryl ester transfer protein (CETP) and triglyceride-rich lipoproteins.Factors that influence the transport of the cholesterol ester to apoB-containing lipoproteins are known to be atherogenic. In an *in vitro* model, incubation of Caco-2 cells with butyrate increased the transcriptional activity of the liver X-receptor. The authors suggest that butyrate from the intestinal fermentation of pectin protects mice from the progression of diet-induced atherosclerosis in apoE$^{-/-}$ mice (Chen *et al.,* 2018). It is noteworthy that HDL-C and ApoAI particles (HDL-C plasma carrier) are responsible for capturing cholesterol from macrophages, promoting reverse cholesterol transport (RCT) to the liver, and cholesterol excretion of bile in feces (Xu *et al.,* 2016). However, pectin could not promote upregulation of level ApoAI mRNA, suggesting that supplementation of this compound may not favor RCT, perhaps the major effect seen is related to inhibition of liver cholesterol synthesis due to downregulation of HMGCR gene expression (Zhu *et al.,* 2013b).However, after not observing a change in the lipogenic targets LXR and ApoAI in the PEC-treated animals, the authors concluded that pectin probably does not reduce hepatic lipogenesis through these pathways. The lipid-rich, customized diet in this study was able to raise cholesterol and alter other parameters such as LDL-C, AST, nitrite and HMGCR, as well as the reduction of ApoA-1 and LXR expression. Paradoxically the diet increased levels of HDL-C, reduced oxidative stress affecting MDA levels, in addition to increasing the gene expression of LCAT and IL-4, an anti-inflammatory cytokine. On the other hand, carnauba-fruit pectin reduced cholesterol, LDL-C, AST, nitrite and HMGCR, showing a beneficial effect in the treatment of dyslipidemia in apoE deficient mice. Although the use of apoE knockout mice is a well-recognized model of dyslipidemia, mouse models are not fully comparable to humans and caution need to be advised regarding the interpretation of our results(Yin *et al.,* 2012). However, altogether our findings support the benefits of carnauba fruit-derived pectin on dyslipidemia with less liver and kidney side effects. More studies using different animal models are warranted to further investigate other metabolic pathways involved in liver cholesterol function and to further support clinical studies in the future.

Conclusion

In conclusion, the results of this study have expanded our current knowledge on the benefits of pectin from carnauba fruits in reducing total cholesterol, LDL-C, glycemia, and body weight, and providing for the first time evidence that carnauba fruit-derived pectin supplementation significantly improved lipid peroxidation and gene expression of HMG-CoA reductase, a key enzyme in endogenous cholesterol synthesis. All these factors contribute to give a further benefit of substances extracted from this plant, with pharmacological properties, nontoxic and underutilized in the pharmaceutical industry and local community, producing a new functional therapeutic product for the prevention of atherosclerosis.

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