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Combined Treatment with Sardine Proteins and *Citrus latifolia* Extract Corrects Dyslipidemia, Prevents Lipid Peroxidation and Improves Lecithin: Cholesterol Acyltransferase and Paraoxonase 1 Activities in Hypercholesterolemic Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author HM, collected and prepared the samples, participated in the laboratory procedures and wrote the first draft of the manuscript. Author DK designed the study supervised the design and analysis of the study. Authors DT and NT participated in the literature searches and analysis of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: In this study, we determine the effects of the supplementation of sardine protein, *Citrus latifolia* extract and the combination of both on lecithin: cholesterol acyltransferase and paraoxonase-1 activities in hypercholesterolemic rats.
Place and Duration of Study: Laboratory of Clinical and Metabolic Nutrition, Department of Biology, Nature and life Sciences Faculty, University of Oran, Algeria, between February and September 2012.
Study Design: Forty male Wistar rats weighing 150-190 g were divided into 4 groups: Hypercholesterolemic (HC) fed a 20% casein diet enriched with cholesterol (1%), HC supplemented with sardine proteins (20%) (HC-*Sp*), HC supplemented with *Citrus latifolia* extract

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(1%) (HC-*Ci*) and HC supplemented with both *Sp* and *Ci* (HC-*Sp*+*Ci*), for 4 weeks.
Methodology: Serum and tissues lipid concentrations were estimated. High density lipoproteins (HDL_2 and HDL_3) were separated and their contents and composition in lipids and apolipoproteins were analyzed. Lecithin: Cholesterol Acyltransferase (LCAT) and paraoxonase 1 (PON 1) activities were determined.
Results: In the HC-*Sp*, HC-*Ci* and HC-*Sp*+*Ci* groups compared with the HC group, plasma, liver, kidney and aorta TC levels were reduced, whereas HDL-cholesterol was increased. In addition, plasma and HDL-TG concentrations were decreased. However, in tissues, TG contents were lower only in the HC-*Sp*+*Ci* group *vs* HC. HDL_2, HDL_3 and tissues lipid peroxidation levels were decreased in HC-*Sp*, HC-*Ci* and HC-*Sp*+*Ci* *vs* HC. In the hypercholesterolemic rats fed with *Sp*, *Ci* extract and *Sp*+*Ci*, LCAT activity was increased compared to HC group. Furthermore, PON 1 activity was elevated in HC-*Ci* and HC-*Sp*+*Ci* group *vs* HC.
Conclusion: In hypercholesterolemic rats, the supplementation with sardine protein, *Citrus latifolia* extract and the combination of both ameliorates tissues and plasma lipid profile and decreases lipid peroxidation in tissues and lipoproteins. Moreover, it improves reverse cholesterol transport by enhancing LCAT activity leading to anti-atherogenic effects. This effect is partially enhanced in HC-*Ci* and HC-*Sp*+*Ci* groups by a high PON 1 activity which protects lipoproteins from oxidation.

Keywords: Sardine protein; Citrus latifolia; High cholesterol diet; LCAT; PON 1.

1. INTRODUCTION

Atherosclerosis is the major contributor to the pathogenesis of heart and vascular diseases [1]. Elevated blood concentration of cholesterol, especially in LDL, constitutes the primary risk factor for atherosclerosis and endothelial dysfunction [2]. Hypercholesterolemia results in free radical production and thereby elevates lipid peroxides.

Fish is one of the main components of a healthy diet and many epidemiologic studies and clinical trials have indicated its beneficial effects in incidence of coronary diseases, by decreasing coronary heart disease (CHD) mortality risk. Many studies from a variety of countries have also reported that seafood consumption protects against lifestyle-related diseases. Numerous epidemiological studies have examined the relationship between dietary marine products and cardiovascular disease (CVD) [3]. In one report, individuals who consumed fatty fish had a 34% reduction in CVD in a three-cohort study [4], and 35 g/day of fish consumption resulted in decreased CVD mortality [5]. A meta-analysis revealed that individuals who consumed fish once a week had a 15% lower risk of CVD mortality compared with individuals who consumed no fish [6]. A previous study suggested that dietary fish protein decreased serum cholesterol through the inhibition of cholesterol and bile acid absorption and the enhancement of cholesterol catabolism in the liver [7].

Citrus is the most important fruit tree crop in the world, with an annual production of approximately 102 million tons [8]. Algeria and many of Mediterranean countries were well known for their important production of *Citrus* fruits. The beneficial effects of flavonoids on human health are universally accepted nowadays for the time being, the main source of flavonoids intake remains dietary intake [9]. Moreover, flavonoids show strong antioxidant and radical scavenging activity and appear to be associated with reduced risk for certain chronic diseases, the prevention of some cardiovascular disorders and certain kinds of cancerous processes [10]. *Citrus* fruits and juices stand out among the most common phenolic rich dietary sources [11]. In recent years, more attention has been paid to the phenolic compounds of *Citrus* fruits, and some publications have suggested that they might play an important role in the antioxidant capacity of *Citrus* fruits [12]. Boshtam et al. [13] studied the effect of lemon on LDL and Ox-LDL affinity to their receptors in rabbits on atherogenic diet. It was concluded that both *Citrus limon* peel and juice can induce LDL and reduce Ox-LDL affinity to related receptors in endothelial cells. As a result of the suggestion by certain epidemiological studies that antioxidant consumption can positively influence health, breeders have recently shown greater interest in developing *Citrus* fruit with higher antioxidant activity [14]. In Algeria and in many Mediterranean countries, major quantities of the peel are not further processed. Some attempts were made to use these residues as livestock feed, although their low nutritional value [15].

HDL plays an important protective role against atherosclerosis and CVD [16]. The anti–atherogenic properties of HDL include the promotion of cellular cholesterol efflux and reverse cholesterol transport (RCT), as well as antioxidant, anti-inflammatory and anti–coagulant effects [17].

Lecithin: Cholesterol acyltransferase (LCAT, EC 2.3.1.43) is a 63–kDa glycoprotein produced exclusively in the liver. This plasma enzyme circulates in association with HDL, in particular HDL_3. It esterifies unesterified cholesterol (UC) to cholesteryl ester (CE) and requires for its activity the apolipoprotein (apo) A-1, cofactor-activator [18]. Paraoxonase 1 (PON 1) is also an enzyme associated with HDL and is implied in the antioxidant capacity of this lipoprotein [19].

The Mediterranean diet is a healthy diet which includes fish, vegetables, fruit, whole grains, legumes, olive oil, and less red meat and dairy products. However, there are no published studies on the effects of sardine proteins combined with lime (*Citrus latifolia*) on experimental hypercholesterolemia induced in the rat. In our investigation sardine (*Sardina pilchardus*) was selected because this fish had a low-cost and is highly consumed in Algeria and *Citrus latifolia* have been used as an edible fruit and a traditional medicine since ancient times. Additionally, citrus lemon juice, rind, and zest are used in a wide variety of food and in marinades for fish. Therefore, the present investigation was designed to evaluate whether sardine proteins, *Citrus latifolia* extract or the combination of both might improve lipid profile and oxidative damages as well as LCAT and PON 1 activities in rats fed a high-cholesterol diet.

2. MATERIALS AND METHODS

2.1 Preparation of *Citrus latifolia* Peels Aqueous Extract

Lime (*Citrus latifolia,* Rutaceae) was purchased from a local market (Oran, Algeria). The *Citrus latifolia* plant was identified taxonomically and authenticated by the Botanical Research Laboratory of Oran University (voucher specimen number CL1965). *Citrus latifolia* peels were minced into small pieces, dried at ambient temperature (24°C) for 7 days and grounded into a fine powder. Then, 500 mL of distilled water was added to 50 g of *Citrus latifolia* and the mixture was heated under reflux for 30 min. The decoction was filtered with cotton wool. The filtrate was concentrated at 65°C by a rotavapor (Buchi Labortechnink AG, Postfach, Switzerland) under a reduced pressure and frozen at -70°C before lyophilization (Christ, alpha 1-2 LD). The crude yield of the lyophilized aqueous extract was approximately 18% (wt/wt). The extract was stored at ambient temperature until use.

2.2 Sardine Protein Process

Fish proteins were prepared from fresh sardines (*Sardina pilchardus*) obtained from a local fish market (Oran, Algeria) according to the method of Guillaume et al. [20]. The general principle consists in separating water and oil from the dry matter. Head, scales, guts and backbone were removed from fresh sardine and the fillets were collected and heated in an oven at 80–85°C (Tan Steril Snc, Fino Mornasco, Italy) for 20 min. At this step, the first separation takes place between a solid phase (coagulated proteins) and a liquid phase (water and oil). A pressing completed this operation; the cake obtained was crumbled and dried at 40–45°C for 24 h and ground in a grinder (IKA, model A116, Hamburg, Germany). The pressurized water was illustrated and centrifuged at 3000 g (Eppendorf, Centrifuge 5702, Hamburg, Germany) to separate oil and the soluble that contained the remained proteins was incorporated with the cake. After crushing, to remove the lipids and other organic solvent soluble elements, the sardine proteins were washed with hexane (Biochem Chemopharma, Montreal, Quebec, Canada) in an extractor of lipids (Soxhlet, Labo-Tech LT.6, Muttenz, Switzerland) for 5 h at 50°C. Sardine protein concentrations were estimated by their nitrogen contents, using mineralization followed by coloration with Nessler's reactive agent and ammonium nitrate as a standard. Then the values were multiplied by 6.25 to obtain protein content which represented 95% in sardine proteins.

2.3 Animals and Treatments

Forty male Wistar rats (Animal Research Center, Pasteur Institute, Algiers, Algeria) weighing 170±20 g were housed in stainless steel cages at temperature of 23±1°C with a 12-hours light/dark cycle and relative humidity of 60%. All the rats received a standard diet containing 20% casein and 1% cholesterol, for 15 days. After this adaptation phase, cholesterolemia value was greater than 5 mmol/L and hypercholesterolemic rats were divided into 4 groups (n=10): Hypercholesterolemic group (HC),

hypercholesterolemic group supplemented with 20% sardine protein (HC-*Sp*), hypercholesterolemic group supplemented with 1% *Citrus latifolia* extract (HC-*Ci*) and hypercholesterolemic group supplemented with the combination of both *Sp* and *Ci* (HC-*Sp*+*Ci*), for 4 weeks. Above Table 1 shows the detailed composition of the diets. Food and tap water were provided *ad libitum*. Food consumption and weight gain were measured every three days. All animal procedures were in strict accordance with the NIH (No. 85-23, revised 1985) Guide for the Care and Use of Laboratory Animals and all experiments have been examined and approved by our institutional committee for animal care and use (UOB 314/2012).

2.3.1 Blood and tissue samples

After 4 weeks of the experiment and overnight fasting, 10 rats from each group were anesthetized with sodium pentobarbital (60 mg/kg body weight) and euthanized with an overdose. Blood was obtained from the abdominal aorta and collected into tubes containing ethylenediaminetetraacetic acid-Na_2 (Sigma, St Louis, MO, USA). Plasma was prepared by low speed centrifugation at 1000g for 20 min at 4°C. Liver, aorta and kidney tissues were removed immediately, rinsed with cold saline solution (0.9%) and weighed. Aliquots of plasma and 50 to 100 mg of tissues were stored at -70°C until analysis.

2.3.2 Isolation and characterization of plasma LDL-HDL_1, HDL_2 and HDL_3

Plasma LDL-HDL_1 was isolated by precipitation using $MgCl_2$ and phosphotungstate (Sigma Chemical Company, France) by the method of Burstein et al. [21]. Plasma HDL_2 and HDL_3 were performed by differential dextran sulphate magnesium chloride precipitation according to Burstein et al. [22]. To estimate the validity of this method, ultracentrifugation was performed according to Havel et al. [23].

Total cholesterol (TC), triglycerides (TG), phospholipids (PL) and unesterified cholesterol (UC) of each HDL_2 and HDL_3 fraction were determined by enzymatic colorimetric methods (kit Biocon Diagnostik Vöhl/Marienhagen, Germany, kit cypress, Langdorp, Belgium, kit CHOD-PAP Wako Chemicals, Richmond, VA, USA).

Table 1. Ingredient composition of the diets fed to rats (g/100 diet)[1]

Ingredients	HC	HC-*Sp*	HC-C*i*	HC-*Sp*+*Ci*
Casein[2]	20	-	20	-
Purified sardine proteins[3]	-	20	-	20
Corn starch[4]	58.5	58.5	57.5	57.5
Sunflower oil[5]	5	5	5	5
Sucrose[6]	4	4	4	4
Cellulose[2]	5	5	5	5
Vitamin mix[7]	2	2	2	2
Mineral mix[8]	4	4	4	4
Cholesterol[9]	1	1	1	1
Cholic acid[9]	0.5	0.5	0.5	0.5
Citrus latifolia extract[3]	-	-	1	1

*[1]Diets were isoenergetic (1.628 MJ/100g diet) and given in powdered form, [2]**PROLABO**, Paris, France. [3]Prepared in our laboratory as previously described. [4]**ONAB**, Sidi Bel Abbès, Algeria. [5]**CEVITAL SPA**, Béjaïa, Algeria. [6]**ENASUCRE**, Sfisef, Algeria. [7]**UAR 200** (Villemoisson, 91360). Vitamin mixture provided the following amounts (mg/kg diet): vitamin A, 39 600 UI; vitamin D_3, 5000 UI; vitamin B_1, 40; vitamin B_2, 30; vitamin B_3, 140; vitamin B_6, 20; vitamin B_7, 300; vitamin B_{12}, 0.1; vitamin C, 1600; vitamin E, 340; vitamin K, 3.80; vitamin PP, 200; choline, 2720; folic acid, 10; paraaminobenzoic acid, 180; biotin, 0.6; and cellulose, qsp, 20 g. [8]**UAR 205B** (Villemoisson, 1360, Epinay/S/Orge, France). Mineral mixture provided the following amounts (mg/kg diet): $CaHPO_4$, 17 200; KCl, 4000; NaCl, 4000; MgO_2, 420; $MgSO_4$, 2000; Fe_2O_3, 120; $FeSO_4$, $7H_2O$, 200; $MnSO_4$, H_2SO_4, H_2O, 98; $CuSO_4$, $5H_2O$, 20; $ZnSO_4$, 80; $CuSO_4$, 80; $CuSO_4$, $7H_2O$; and KI, 0.32, [9]**Merck**, Darmstadt, Germany. **HC**: hypercholesterolemic diet, **HC-Sp**: hypercholesterolemic diet supplemented with sardine proteins, **HC-Ci**: hypercholesterolemic diet supplemented with Citrus latifolia, **HC-Sp+Ci**: hypercholesterolemic diet supplemented with the combination of **Sp+Ci***

Esterified Cholesterol (EC) concentrations were obtained by calculating the difference between TC and UC values. Cholesteryl esters (CE) levels were estimated as 1.67 times the esterified cholesterol content.

Apolipoprotein concentrations of HDL_2 and HDL_3 were measured according to the method of Lowry et al. [24], using bovine serum albumin (Sigma Chemical Company, St Louis, USA) as standard.

2.3.3 Lipid peroxidation

Thiobarbituric Acid Reactive Substances (TBARS) concentrations of plasma lipoprotein were measured according to the method of Quintanilha et al. [25]. One milliliter of lipoprotein fraction was added to 2 mL of thiobarbituric acid (TBA) (final concentration, 0.017 mmol/L), plus

butylated hydroxytoluene (concentration, 3.36 µmol/L) and incubated for 15 min at 100°C. After cooling and centrifugation, the absorbance of the supernatant was measured as 535 nm. Lipid peroxidation in tissues was assessed by the complex formed between malondialdehyde and thiobarbituric acid (TBA) [26]. Briefly, liver, aorta and kidney tissues (0.5 g) were homogenized with 4.5 mL of KCl (1.15%). The homogenate (100 µL) was mixed with 0.1 mL sodium dodecylsulfate (SDS) (8.1%), 750 µL acetic acid (20%) and 750 µL TBA reagent (0.8%). The reaction mixture was heated at 95°C for 60 min. After heating, the tubes were cooled and 2.5 mL of *n*-butanol-pyridine (15:1) was added. After mixing and centrifugation at 4000 g for 10 min, the upper phase was taken for measurement at 532 nm.

2.3.4 Assay for LCAT activity

LCAT activity was determined on fresh plasma by an endogenous method [27]. This procedure was based on the disappearance of the UC molecules which were transformed into EC after 4 h of incubation at 37°C, starting from a fatty acid and lecithin. UC was evaluated by the method described previously. The cholesterol esterifying activity was expressed as nanomoles of esterified cholesterol/mL/h of plasma.

$$\text{LCAT activity} = (\text{UC } t_{0h} - \text{UC } t_{4h})/4\text{h of incubation.}$$

2.3.5 Assay for PON 1 activity

Paraoxonase activity measurement was performed in the absence (basal activity) and presence of NaCl (salt-stimulated activity). The rate of paraoxon hydrolysis (diethyl–*p*–nitrophenylphosphate) was measured by monitoring the increase of absorbance at 270 nm and 25°C. For this assay, 10 µL of sample was added to 1 mL of a solution containing 10 mM of phenyl acetate in 20 mM Tris/HCl pH 8.0 and 1 mM $CaCl_2$. The activity was calculated relative to the molecular extinction coefficient of the phenyl (1310 M^{-1} cm^{-1}) [28].

2.3.6 Statistical analysis

All quantitative measurements were expressed as means ± standard deviations (SD) for ten rats/group. Data were analyzed using one-way analysis of variance (ANOVA) followed by post hoc Dunnett´s multiple-comparison test using Statistica software (version 9.0, Stat Soft, Inc., Tulsa, OK, USA). *P = .05* was considered statistically significant.

3. RESULTS

3.1 Body Weight (BW), Food Intake and Relative Organ Weights

After 4 weeks of the experiment, BW was decreased by 19% and 13%, respectively in the hypercholesterolemic groups treated with *Citrus latifolia* (*Ci*) lyophilised aqueous extract and the combination of both sardine protein (*Sp*) and *Ci* (*Sp*+*Ci*) compared with the untreated group. Furthermore, a decrease in food intake was noted in these groups (-18% and -13%, respectively in HC-*Ci* and HC-*Ci*+*Sp vs* HC) (Table 2). However, there was no significant difference in BW and food intake in the HC-*Sp* group than in the HC group. The relative weights of liver, aorta and kidney were similar in all the groups.

3.2 Plasma and Tissue Lipid Concentrations

Table 3 shows that in rats fed the hypercholesterolemic diets supplemented with sardine protein (*Sp*), *Citrus latifolia* (*Ci*) lyophilized aqueous extract or the combination of both (*Sp*+*Ci)*, plasma total cholesterol (TC) levels were respectively reduced by 38%, 36% and 51% than in untreated HC rats. Moreover, liver, aorta and kidney TC contents were significantly decreased ($p<0.05$). However, HDL-C concentrations were respectively 1.8-, 1.6- and 1.8-fold higher in HC-*Sp*, HC-*Ci* and HC-*Sp*+*Ci* groups than in HC.

Plasma and HDL triacylglycerols (TG) levels were significantly lower with the hypercholesterolemic diets supplemented with *Sp*, *Ci* and *Sp*+*Ci* compared to the hypercholesterolemic diet alone (*P = .05*). Nevertheless, sardine protein and *Citrus latifolia* supplementation did not affect liver, aorta and kidney TG contents, whereas the combination *Sp*+*Ci* decreased them by 48% and 21%, respectively in liver and aorta.

3.3 Tissues and Lipoproteins Lipid Peroxidation Levels

In HC-*Sp*, HC-*Ci* and HC-*Sp*+*Ci vs* HC group, TBARS values were decreased respectively by 50%, 54% and 83% in liver, 67%, 72% and 53%

in aorta and 72%, 76% and 84% in kidney (Table 4).

In lipoproteins, TBARS levels were reduced in HDL_2, HDL_3 and LDL-HDL_1 of HC-*Sp* and HC-*Sp*+*Ci* groups *vs* HC. Moreover, HDL_2 and LDL-HDL_1 TBARS were 2- and 1.6-fold lower in HC-*Ci vs* HC.

3.4 HDL_2 and HDL_3 Apolipoprotein and Lipid Contents

Compared with the HC group, HDL_3-PL (LCAT substrate) contents were respectively decreased by 29%, 44% and 28% in HC-*Sp,* HC-*Ci* and HC-*Sp*+*Ci* groups (Table 5). Moreover, HDL_3-UC (acyl group acceptor) values of the rats fed HC-*Sp* and HC-*Ci* diets were diminished respectively by 40% and 35% as compared to HC. Furthermore, HDL_3-TG and HDL_3-CE were significantly reduced (*P = .05*). In HDL_2, CE concentrations (product of LCAT reaction) were respectively increased by 14%, 17% and 12% in HC-*Sp,* HC-*Ci* and HC-*Sp*+*Ci* groups compared with the HC group. In addition, in HC-*Sp,* HC-*Ci* and HC-*Sp*+*Ci* groups compared to HC group, HDL_2-TG and HDL_2-UC were significantly reduced, whereas HDL_2-PL contents were respectively 1.4, 1.6- and 1.5-fold higher.

3.5 LCAT and PON 1 Activities

In HC-*Sp*, HC-*Ci* and HC-*Sp*+*Ci* groups, LCAT activity was respectively 1.7-, 1.5- and 2.5-fold higher compared to HC values (Table 6). Furthermore, PON 1 activity was increased by 13% and 32%, respectively in HC-*Ci* and HC-*Sp*+*Ci* groups *vs* HC.

Table 2. Body Weight (BW), food intake and relative organ weights

	HC	HC-*Sp*	HC-C*i*	HC-*Sp*+*Ci*
BW (g)	217.50±3.3	222.70±3.8	176.90±4$^{\#}$	189.13±3$^{\$}$
Food intake (g/d/rat)	23.33±1.0	23.31±1.1	9.11±0.3$^{\#}$	20.26±1.0$^{\$}$
Relative organ weights				
Liver **(**g/100 g)	4.07±0.4	4.13±0.6	3.91±0.4	4.28±0.8
Aorta (g/100 g)	0.05±0.01	0.02±0.01*	0.03±0.01	0.03±0.01
Kidney (g/100 g)	0.79±0.3	0.80±0.3	0.75±0.2	0.72±0.2

Values were given as means ± SD. of 10 rats per group. The results were considered statistically significant if the P = .05. ***HC****: hypercholesterolemic diet,* ***HC-Sp****: hypercholesterolemic diet supplemented with sardine proteins,* ***HC-Ci****: hypercholesterolemic diet supplemented with Citrus latifolia,* ***HC-Sp+Ci****: hypercholesterolemic diet supplemented with the combination of* ***Sp+Ci,*** **HC-Sp vs HC group, $^{\#}$HC-Ci vs HC group, $^{\$}$HC-Sp+Ci vs HC group,* ***Relative organ weight****=Total organ weight/Final body weight x 100*

Table 3. Concentrations of total Cholesterol (TC) and Triacylglycerols (TG) in plasma, HDL fraction, liver, aorta and kidney

		HC	HC-*Sp*	HC-C*i*	HC-*Sp*+*Ci*
	Plasma (mmol.L^{-1})	5.93±0.2	3.69±0.3*	3.76±0.2$^{\#}$	2.88±0.2$^{\$}$
	HDL (mmol.L^{-1})	1.33±0.1	2.39±0.1*	2.11±0.1$^{\#}$	2.36±0.1$^{\$}$
TC	*Liver (µmol.g^{-1})*	135.59±5.7	85.16±3.4*	82.61±4.3$^{\#}$	71.22±2.2$^{\$}$
	Aorta (µmol.g^{-1})	124.15±3.2	72.77±5.4*	68.23±2.5$^{\#}$	60.20±3$^{\$}$
	Kidney (µmol.g^{-1})	98.23±3.2	60.22±3.2*	56.21±2.5$^{\#}$	51.82±2$^{\$}$
	Plasma (mmol.L^{-1})	1.84±0.01	0.88±0.2*	0.95±0.1$^{\#}$	0.61±0.01$^{\$}$
	HDL (mmol.L^{-1})	1.01±0.1	0.39±0.04*	0.56±0.04$^{\#}$	0.27±0.02$^{\$}$
TG	*Liver (µmol.g^{-1})*	85.66±10.3	82.40±11.4	83.40±12.5	44.08±10.2$^{\$}$
	Aorta (µmol.g^{-1})	75.41±6.2	71.53±3.5	68.27±5.4	59.21±2.5$^{\$}$
	Kidney (µmol.g^{-1})	58.23±5.2	55.35±4.21	54.68±5.5	55.41±5.1

*Values were given as means ± SD of 10 rats per group. The results were considered statistically significant if the P = .05, *HC-Sp vs HC group, $^{\#}$HC-Ci vs HC group, $^{\$}$HC-Sp+Ci vs HC group*

Table 4. Thiobarbituric Acid Reactive Substances (TBARS) contents of tissues and lipoproteins

		HC	HC-*Sp*	HC-C*i*	HC-*Sp*+*Ci*
Tissues (mmol/100g)	*Liver*	1.29±0.15	0.64±0.11*	0.59±0.06$^{\#}$	0.21±0.01$^{\$}$
	Aorta	1.36±0.10	0.45±0.02*	0.38±0.02$^{\#}$	0.64±0.03$^{\$}$
	Kidney	1.14±0.12	0.32±0.13*	0.27±0.01$^{\#}$	0.18±0.01$^{\$}$
Lipoproteins (nmol/mL)	*HDL_2*	3.15±0.20	2.83±0.31*	1.47±0.27$^{\#}$	1.24±0.21$^{\$}$
	HDL_3	1.77±0.33	1.11±0.14*	1.52±0.17	1.04±0.18$^{\$}$
	LDL- HDL_1	2.57±0.12	1.17±0.10*	1.60±0.11$^{\#}$	1.44±0.13$^{\$}$

Values were given as means ± SD of 10 rats per group. The results were considered statistically significant if the P = .05. ***C****: hypercholesterolemic diet,* ***HC-Sp****: hypercholesterolemic diet supplemented with sardine proteins,* ***HC-Ci****: hypercholesterolemic diet supplemented with Citrus latifolia,* ***HC-Sp+Ci****: hypercholesterolemic diet supplemented with the combination of* ***Sp+Ci,*** **HC-Sp vs HC group. $^{\#}$HC-Ci vs HC group, $^{\$}$HC-Sp+Ci vs HC group*

Table 5. Plasma HDL_2 and HDL_3 apolipoproteins (apos) (g/L) and lipids (mmol/L) levels

Lipoproteins	HC	HC-*Sp*	HC-C*i*	HC-*Sp*+*Ci*
HDL_2				
Apos	0.050±0.007	0.059±0.002	0.051±0.006	0.058±0.002
TG	0.54±0.02	0.19±0.01*	0.28±0.09$^{\#}$	0.13±0.08$^{\$}$
PL	0.21±0.04	0.30±0.01*	0.34±0.02$^{\#}$	0.31±0.01$^{\$}$
UC	0.34±0.02	0.21±0.02*	0.22±0.01$^{\#}$	0.17±0.04$^{\$}$
CE	0.74±0.11	0.86±0.05*	0.89±0.06$^{\#}$	0.84±0.06$^{\$}$
HDL_3				
Apos	0.22±0.003	0.27±0.007*	0.24±0.001	0.21±0.03
TG	0.43±0.01	0.16±0.01*	0.23±0.07$^{\#}$	0.08±0.001$^{\$}$
PL	0.90±0.011	0.64±0.03*	0.50±0.08$^{\#}$	0.65±0.08$^{\$}$
UC	0.20±0.06	0.12±0.06*	0.13±0.01$^{\#}$	0.19±0.02
CE	1.59±0.05	0.99±0.08*	1.01±0.04$^{\#}$	0.77±0.03$^{\$}$

*Values were given as means ± SD. of 10 rats per group. The results were considered statistically significant if the P = .05, *HC-Sp vs HC group, $^{\#}$HC-Ci vs HC group, $^{\$}$HC-Sp+Ci vs HC group*

Table 6. Lecithin: cholesterol acyltransferase (LCAT) and paraoxonase 1 (PON 1) activities

	HC	HC-*Sp*	HC-C*i*	HC-*Sp*+*Ci*
LCAT (nmol/mL/h)	45.80±5.1	78.55±12.4*	70.99±13.0$^{\#}$	95.50±12.0$^{\$}$
PON 1 (UI/mL)	17.24±1.2	17.04±1.3	19.90±1.6$^{\#}$	25.32±1.5$^{\$}$

Values were given as means ± SD of 10 rats per group. The results were considered statistically significant if the P = .05. ***C****: hypercholesterolemic diet,* ***HC-Sp****: hypercholesterolemic diet supplemented with sardine proteins,* ***HC-Ci****: hypercholesterolemic diet supplemented with Citrus latifolia,* ***HC-Sp+Ci****: hypercholesterolemic diet supplemented with the combination of* ***Sp+Ci,*** **HC-Sp vs HC group, $^{\#}$HC-Ci vs HC group, $^{\$}$HC-Sp+Ci vs HC group*

4. DISCUSSION

In the present study, the hypercholesterolemic diet supplemented with sardine proteins (HC-*Sp*) compared to the hypercholesterolemic diet (HC) showed similar body weight and food intake. Our data could suggest that sardine protein was well accepted by the rats since it induced a normal body growth. This result was in agreement with the previous study of Bastida et al. [29]. Nevertheless, the rats fed the HC diet supplemented with *Citrus latifolia* (*Ci*) aqueous extract or with the combination of both *Ci* extract and *Sp* exhibited lower body weights compared with those fed the HC diet. Indeed, food intake was decreased in these groups and this could probably be due to a lesser palatability of *Citrus latifolia* (*Ci*) aqueous extract than fish protein.

Hypercholesterolemia is a major risk factor for cardiovascular diseases and feeding experimental rodents with high cholesterol diets (HCD) is reported to cause hypercholesterolemia and cholesterol deposit in liver [30]. The liver is the main organ responsible for cholesterol transport, metabolism and excretion, so it is

reasonable to study hepatic lipemic-oxidative disorders in high cholesterol diet induced atherogenesis.

In our study, the hypercholesterolemic groups treated with sardine proteins, *Citrus latifolia* extract or the combination of both (*Sp+Ci*) showed a decrease in plasma TC and inversely an increase in HDL-C levels. Ben Khaled et al. have demonstrated a decrease in plasma TC in rats fed a high cholesterol diet supplemented with sardine proteins [31]. Furthermore, Khan et al. have reported the hypolipidemic effects of *Citrus* lemon which contained important natural components, including citric acid, vitamin C, minerals and flavonoids although health-related properties associated with their content of vitamin C [32]. Consequently, in our study, the hypocholesterolemic effect obtained with *Citrus latifolia* supplementation may be due to the antioxidant effects of vitamin C and flavonoids, which would improve the endothelial function of coronary and peripheral vessels. The hypocholesterolemic effect was more pronounced in the hypercholesterolemic rats fed the combination *Sp-Ci* indicating a possible synergistic action of sardine proteins and *Citrus latifolia* components.

Another aspect of the role of fish proteins in human health pertains to their possible effects on lipid metabolism. In this context, several investigators have demonstrated that fish proteins affect serum cholesterol levels in experimental animals [33]. A previous study suggested that dietary fish protein decreased serum cholesterol through the inhibition of cholesterol and bile acid absorption and the enhancement of cholesterol catabolism in the liver [7]. Furthermore, an association with *Citrus* flavonoids, including naringenin, hesperidin, nobiletin and tangeretin, have emerged as promising therapeutic agents for the treatment of metabolic dysregulation. It has also been reported that hesperidin has the ability to inhibit copper-induced low density lipoprotein (LDL) oxidation [34]. In mouse models, *Citrus* flavonoids supplements prevent hepatic steatosis, dyslipidemia primarily through inhibition of hepatic fatty acid synthesis and increased fatty acid oxidation in metabolically important tissues including liver, adipose tissue, kidney and aorta [35]. Modified LDL was not recognized by the LDL-receptors apo (B/E), but instead, it is taken up in a non-regulated manner through scavenger receptors present in the surface of monocytes and macrophages. This process leads to the accumulation of esterified and unesterified cholesterol in the macrophages and, consequently, to the formation of foam cells, the hallmark of the atherosclerotic lesion.

The high cholesterol diets supplemented with *Sp*, *Ci* and both *Sp+Ci* significantly reduced liver total cholesterol compared with the high cholesterol diet alone. These results possibly suggest an inhibition of cholesterol biosynthesis by the liver and an interruption of the enterohepatic circulation of cholesterol and its metabolites which might be responsible for the plasma cholesterol-lowering action of these diets. Numerous studies in animals fed a high cholesterol diet indicated that the mechanisms which prevent dyslipidemia in rats are due to the decline of the feedback inhibition of cholesterol biosynthesis and raised bile acid excretion leading to a minor elevation of serum cholesterol levels [36].

The hepatic TG level is controlled mainly by TG synthesis, beta-oxidation and secretion in the form of lipoprotein [37]. The combination *Sp-Ci* caused a significant reduction in liver TG concentrations. This result indicated an inhibition of lipid biosynthesis which might be the possible mechanism of its lipid-lowering action. It has also been suggested that lipid and protein oxidation leads to impaired lipid transport and cell injury, and consequently contributes to the development of hypercholesterolemia [38]. In addition, a mixture of naringin and hesperidin significantly lowered the levels of plasma and hepatic cholesterol and triglycerides as well as hydroxy-methyl-glutaryl-CoA reductase (HMG-CoA reductase) activity in rats [39]. Plasma TG has been shown to decrease with fish intake in many studies. It is believed that this effect is also mainly mediated by fish n-3 polyunsaturated fatty acids (PUFA) contents.

Lipid peroxidation is a free radical chain reaction, which is triggered by hydroxyl radical and leads to membrane breakdown producing more free radicals [40]. In our study, feeding hypercholesterolemic rats with sardine proteins or *Citrus latifolia* extract or the combination of both produced a significant decrease in tissues and lipoproteins lipid peroxidation products. A reduction in TBARS levels in liver and aorta was noted in hypercholesterolemic rats fed 20% sardine proteins [38]. In addition, the study of Ben Khaled et al. [31] reported that the incorporation of S*ardinella aurita* protein into a cholesterol enriched diet improved antioxidant

activity and lowered lipid peroxidation in Wistar rats. The beneficial effect of sardine protein on dyslipidemia and oxidative stress is partly due to its high taurine content [41]. Indeed, this amino-acid has demonstrated antihypertensive, hypocholesterolemic and antioxidant properties. In hypercholesterolemic rabbits, Bolayirli et al. showed a significant increase of susceptibility to free radical-induced lipid peroxidation [42]. Hamza-Reguig et al. have shown an increase in TBARS levels in liver, heart and aorta of hypercholesterolemic rats compared with controls [43]. Lemon fruits, rich in flavonoids are a very important part of a balanced diet, particularly for their role in cardiovascular disease prevention [8]. The peel is rich in hesperidin an important flavonoid in *Citrus* and the principal flavanone in lime and other *Citrus* species. It is an effective antioxidant since it is able to quench the oxygen free radicals. This flavone has anti-lipid peroxidation properties and protects against free radical damage [44].

Vitamin C is well known for its strong ability to prevent oxidative damage and to scavenge free radicals such as reactive oxygen species (ROS) effectively produced through biological processes in many extracellular and intracellular reactions [45]; this property may be through induction of endogenous antioxidants in HCD animal models. In the present study, HCD induced hepatic oxidative injury was ameliorated by combining HCD with sardine proteins (*Sp*) or *Citrus latifolia* (*Ci*) extract and this improvement was more significant with the combined treatment (*Sp*+*Ci*).

LCAT is a major modulator of HDL metabolism. Our results showed that LCAT activity was significantly higher in rats fed hypercholesterolemic diets supplemented with sardine proteins, *Citrus latifolia* extract or the combination of both (*Sp*+*Ci*) compared to the hypercholesterolemic group. Moreover, HDL_2-cholestery esters (HDL_2-CE) content (product of enzymatic reaction) was increased. However, a reduction in HDL_3-phospholipids (substrate of LCAT) and HDL_3-UC (acceptor of lecithin acyl groups) was noted. The enhanced LCAT activity could possibly be related to an increased expression of its apo AI cofactor activator and/or its hepatic genes synthesis in the liver. This assumption is supported by Shukla et al. [33] who showed that dietary fish protein compared to casein had a higher relative hepatic gene involved in the synthesis of apo AI and LCAT. Feeding sardine proteins enhanced probably an efficient flow of free cholesterol from peripheral tissues to plasma *via* the HDL_2 rich in cholesteryl esters. Indeed, the higher level of HDL_2-CE could probably create a gradient necessary for this transfer, involving thus a high efficacy of reverse cholesterol transport.

Some reports have mentioned that LCAT enzyme may have a dual role in lipoprotein oxidation, whereby it acts in a dose-responsive manner as a potent prooxidant during VLDL oxidation, but as an antioxidant during LDL oxidation [46]. Ansell et al. [47] have reported that HDL_3-phospholipids are especially capable of retarding LDL oxidation. Moreover, the low PL content noted in HDL_3 was probably due to the higher LCAT activity. Nevertheless, at the same time this enhanced enzyme activity promoted an efficient cholesterol efflux by HDL-CE rich toward liver for excretion in bile acids form.

Paraoxonase 1 (PON 1) is another antioxidant enzyme closely associated with HDL. It is a calcium-dependent esterase which detoxifies lipid peroxides, and is widely distributed in many tissues. In human studies, higher PON 1 activity is associated with a lower incidence of major cardiovascular events [48]. Moreover, PON 1 prevents the oxidation of the lipoproteins of weak density (LDL) [49]. Our data showed that in hypercholesterolemic rats, the supplementation of both sardine protein diet with *Citrus latifolia* extract decreased LDL-HDL_1 lipid peroxidation and improved PON 1 activity in plasma, protecting thus LDL from oxidation, and consequently seemed to reduce CVD. PON 1 activity could be increased as a consequence of an improved synthesis of HDL secondary to the enhanced LCAT activity. Lee et al. indicated that male rats consuming a high-cholesterol diet (1%) for 6 weeks with naringenin supplements was effective in lowering plasma TBARS levels and enhancing plasma PON 1 activity [50].

5. CONCLUSION

In hypercholesterolemic rats, consuming both sardine proteins (*Sp*) and *Citrus latifolia* (*Ci*) extract ameliorates plasma and liver lipid profiles and reduces lipid peroxidation in lipoproteins (LDL-HDL_1, HDL_2 and HDL_3) and tissues (liver, kidney and aorta). Moreover, the combined treatment *Sp*+*Ci* improves reverse cholesterol transport (RCT) from peripheral tissues to the liver by increasing LCAT activity, thus ensuring a fortification in HDL_2-cholesteryl esters. This anti-atherogenic effect is particularly reinforced by the increase in PON 1 activity, which protects HDL

and LDL from oxidation. When *Sp* and *Ci* were provided in combination to hypercholesterolemic rats, they showed an antioxidant and hypolipidemic effects. These results suggest that both sardine proteins and *Citrus latifolia* are useful in the primary treatment of hypercholesterolemia and oxidative damage caused by a high-cholesterol diet. Further clinical studies are needed to elucidate this hypothesis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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