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Curcumin exerts a protective effect against obesity and liver injury induced by an atherogenic diet

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ABSTRACT

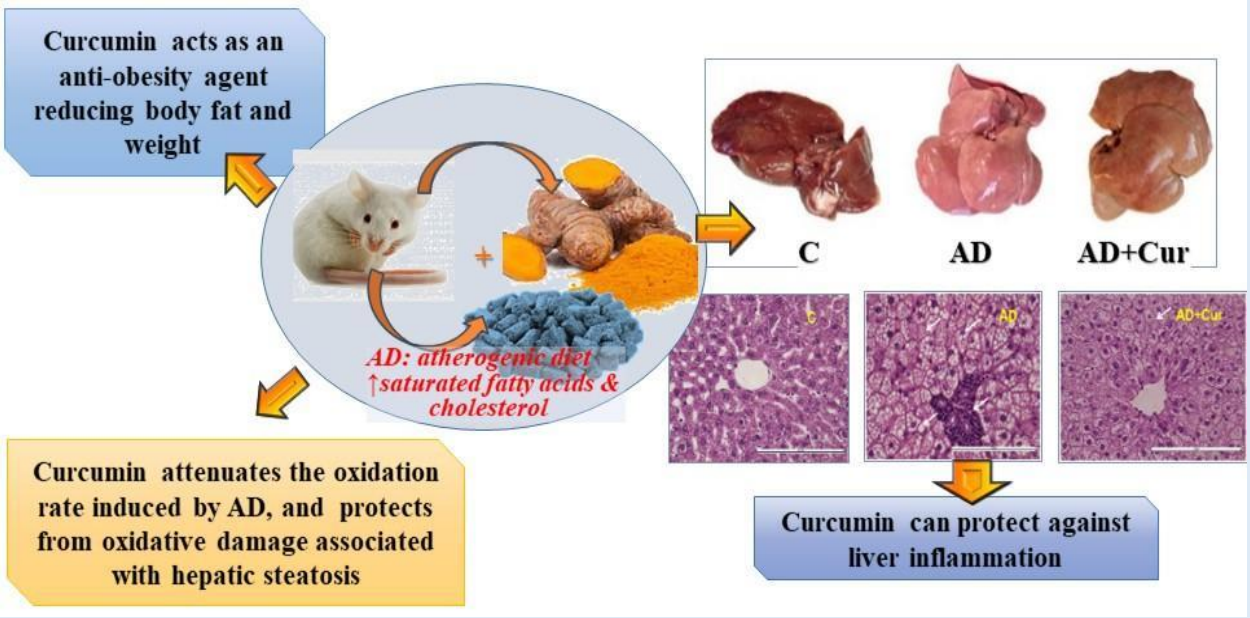
Background: Curcumin (Cur) is a natural yellow polyphenol extracted from the *turmeric* rhizome (*Curcuma longa*). Cur is known for its potential therapeutic properties as an analgesic, anti-inflammatory, antioxidant, antimicrobial, hepatoprotective, and anti-mutagenic, although some of these biological activities remain unproven. Epidemiological studies have shown a positive relationship between high-fat diets and diet-related chronic diseases. We hypothesized that some adverse effects of consuming atherogenic or high-fat diets (AD) can be ameliorated by Cur supplementation. Using an experimental model of rats, this study investigated the significance of Cur when it is given as a supplement in an AD.

Methods: Healthy adult Wistar rats were randomly assigned to one of three groups. Controls (C) received a standard diet and experimental rats were fed with AD or AD+Cur for 5 weeks. Cur (100 mg/kg body weight) was given orally daily, plus piperine (5 mg/kg body weight). The effect of Cur supplementation was studied on zoometrics, visceral fat content, serum lipids profile, hepatosteatosis, liver function and oxidative status.

Results: Diets did not alter energy consumption. As compared to the other groups, AD+Cur group showed a lower total visceral fat content, percentage of perirenal, mesenteric, and pelvic fat, and body weight gain ($P< 0.05$). Serum total cholesterol ($P<0.0001$), non-HDL-C ($P<0.0001$) levels were significantly higher in AD groups as compared with C. Serum triglycerides and HDL-C levels remained similar among groups ($P>0.05$). AD induced a liver injury with macrovesicular steatosis and portal inflammation. AD+Cur rats presented microvesicular steatosis with no inflammation, achieving the lowest level of alanine aminotransferase (ALT; $P<0.0001$) and reductions of aspartate aminotransferase (AST; $P<0.0001$). Liver homogenates from AD+Cur showed that Cur supplementation reduced the dichlorofluorescein diacetate (DCFH-DA) oxidation rate induced by AD by 25 % and deferoxamine and superoxide dismutase inhibited DCFH-DA.

Conclusion: Cur as a dietary supplement showed a protective effect against obesity and inflammation, but its cardioprotective ability remained unproved. Cur may develop as a promising therapeutic agent for liver diseases induced by oxidative stress. This study provides supporting evidence to confirm the beneficial effects of curcumin from the point of view of functional food science.

Keywords: curcumin, liver injury, ROS, atherogenic diet, visceral fat, obesity



INTRODUCTION

In the past few years, the increased sedentary lifestyle and consumption of food rich in saturated fats and cholesterol have exposed the population of developed countries to emerging health problems [1]. The steadily increasing incidence of obesity, cardiovascular disease (CVD) linked to the presence of hyperlipidemia, hypertension, diabetes mellitus, cancers, digestive diseases, and some skeletal, kidney, and liver diseases [2-3] can be mentioned among these emerging health problems. In the Americas, obesity has reached epidemic proportions, and prevalence is higher in women [4]. In addition, it was recently shown that the origin of these diet-related chronic diseases (DRCD) is multifactorial and may result from different deregulated metabolic parameters, including antioxidant status, acid-base imbalance, increased inflammatory status, and impaired carbohydrate/lipid/one-carbon metabolism [3].

Atherogenic and high-fat diets (AD) in animals tend to develop DRCD and liver damage, similar to the phenotype observed in humans with non-alcoholic fatty liver disease (NAFLD) [5]. NAFLD is characterized by fat accumulation in hepatocytes in the absence of alcohol intake. It is estimated that 25% of the world's population has NAFLD [6]. In South America, this figure reaches 30.5% [7]. Non-alcoholic steatohepatitis develops when physiological adaptive mechanisms of the liver are overwhelmed by the excessive influx of triglycerides (TG). This leads to lipotoxicity, inflammation, reactive oxygen species (ROS) formation, and hepatocellular dysfunction [8].

Oxidative stress is generated when ROS formation is not compensated by the antioxidant defense capability or redox signaling is disrupted, affecting cell functionality [9]. This metabolic alteration is an important factor in the pathogenesis of hepatocyte dysfunction [10]. Furthermore, an AD clearly affects the hepatocyte energy metabolism leading to depressed b-oxidation and increased mitochondrial ROS production [11]. Undoubtedly, ROS play a crucial role in the development of numerous chronic liver diseases and stimulate their progression [12].

Natural products represent a source of discovery of new basis for therapeutics guides that can be used in treating different types of DRCD. Patients use herbal medicines due to their wide availability, low toxicity, pharmacological activity, chemical diversity and low side effects compared to synthetic drugs [13]. Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene3,5-dione] (Cur) is a natural yellow polyphenol extracted from the *turmeric* rhizome (*Curcuma longa*), a plant that grows in tropical and subtropical regions throughout the world, which is extensively used for food preparation in many Asian countries [14]. Cur was incorporated into cooking and therapeutic preparations throughout the centuries in different parts of the world and was used as a combined herbal medicine in the treatment of various diseases due to its antioxidant, anti-inflammatory, antimutagenic, antimicrobial and anticancer properties [15-16]. *In vivo* and *in vitro* studies revealed that Cur exerts anti-obesity and anti-inflammatory effects by decreasing adiposity, lipid storage, and increasing the oxidation of fatty acids [17].

We hypothesized that Cur can ameliorate some adverse effects of consuming AD by modulating plasma lipid levels, decreasing body fat mass and preventing liver injury. In an experimental model of rats, this study tested the hypothesis investigating the significance of Cur when it is given as a supplement in an AD. The effect of Cur supplementation was studied on zoometrics, visceral fat content, serum lipids profile, hepatosteatosis, liver function and oxidative status.

METHODS

Animals: Healthy adult female Wistar rats (n=21) with an initial body weight of 200 ± 20 g (aged: 44±2 days), were used in this trial. The animals were obtained from the laboratory of the Department of Biochemistry, Faculty of Dentistry, University of Buenos Aires, Argentina. Animals were housed in galvanized cages with meshed floors to maintain hygienic conditions and avoid coprophagy. Rats were kept in individual cages and exposed to a 12-h light/dark cycle throughout the study. The room temperature was maintained at 21 ± 1 °C with a humidity of 50–60%.

Ethics: This study was approved by the University of Buenos Aires, Argentina by the Ethical Commission of the School of Dentistry, under Protocol (UBACyT 20020150200013BA, Res CD 398/151). The animals were maintained in accordance with the Guide of the National Academy of Science Animal Welfare Regulations [18]. The study was a part of the doctoral thesis of the lead author of the paper (MEA).

Diets: The composition of the diets is shown in Table **1**. The control (C) diet was a standard diet (Purina chow; Gilardoni SA, Buenos Aires, Argentina). The AD used butter as the main source of fat (80% fat) and contained a 15.0% fat/100 g diet; it was rich in saturated fatty acids and cholesterol (1.51 g/100 g diet). All diets had similar grain sizes. The ingredients of the two diets were milled, mixed and the homogenized material was transferred to the press. The diets were manually cut to achieve a size suitable for rodents, similar to the commercial stock diet. Diets were prepared every 2 days and stored at -4°C until fed. Fresh diets were offered daily and food containers were cleaned before being refilled. Food cups were refilled once a day, and food consumption was measured with a Mettler scale PC 4000 (accuracy±1 mg). Daily food intake was recorded as kcal per 100 g of body weight per day (kcal/100 g BW/day).

Table 1. Composition of standard and experimental diet

Ingredients (g/100 g)	C	AD
Energy		
kcal	270	337
kJ	1130	1412
Carbohydrates	40.00	34.00
Protein (mix of corn, wheat, soybean, fish, and meat flour)	19.00	16.00
Total Fat (acid hydrolysis) (mix of corn oil, fish oil, and butter)	4.00	15.00
Saturated fatty acids	1.65	9.00
Monounsaturated fatty acids	1.20	4.20
Polyunsaturated fatty acids n-6	1.12	1.60
Polyunsaturated fatty acids n-3	0.03	0.20
Cholesterol	0.01	1.51
Fiber Crude	5.13	4.50
Ash	8.06	7.10
Vitamin mixture	0.80	0.80
Water	23.00	21.00

C: control standard diet consisted of commercially available pellets (Purina chow). **AD:** atherogenic diet, rich in saturated fat and cholesterol.

Experimental design: Firstly, Rats (n=21) were weighed and numbered according to their body weight. Then, they were assigned into three groups (n=7/group): C, control group, was given access to a standard diet; group fed with AD (AD group), and AD+Cur group were given an AD plus Cur to study the effects of Cur in an AD-fed condition. The composition of the experimental diets is described in the section Diets. The dose of 100 mg/kg body weight (BW)/day of Cur [from *Cúrcuma longa (Tumeric)*, as powder. Sigma Aldrich S.R.L] was calculated according to the bibliography [19] considering the absence of adverse toxicological effects. Cur was co-administered orally with piperine (5 mg/kg BW/day; Sigma Aldrich S.R.L) to enhance Cur's bioavailability [20]. Cur and piperine were dissolved in 0.1% carboxymethyl cellulose (CMC) and given through oral gavage to the animals. Groups C and AD received a daily oral administration of the same volume of the CMC alone.

Throughout the 5 weeks of the experimental period, the animals accessed food and water ad libitum. After 5 weeks of intervention, food and water were removed at the end of the dark period (7:00 AM). After 4 h of fasting, animals were euthanized with an intraperitoneal injection of sodium Thiopental (4 mg/100g BW; Scott-Cassara. Buenos Aires. Argentina). The total carcass was weighed. Blood samples were obtained by cardiac puncture and rapidly centrifuged at 1500g for 15 min. Serum samples were stored at -20°C until biochemical assays were performed. The liver was weighted, one piece was kept at -70ºC (for biochemical assays) and another piece was placed in 10% of formalin for histological evaluation.

Biochemical determinations, hepatic index (HI), histology, and body fat content- distribution were performed in all rats.

Zoometry: Total BW was measured weekly after a fasting period of 2-4 h, which allowed enough time to determine zoometric parameters and others [21]. A Mettler PC 4000 scale (accuracy 60.001 g) was used to measure BW.

Hepatic index determination: The liver was removed immediately after euthanasia to avoid dehydration and was weighed with an electronic analytical scale. The data was used to calculate the hepatic index (HI). The liver weight of each rat was normalized to the percentage of the total BW to minimize the individual differences in body size. This index was expressed as liver weight/total BW x 100 (HI %) [22]. The HI was used to determine enlargement of the liver (hepatomegaly).

Measurement of visceral fat: Fat mass was measured by an analytical balance (Mettler Toledo) and assessed by weighing the perirenal, mesenteric, and pelvic white adipose tissue [23]. The sum of these fat tissues was considered visceral fat. The data were expressed as a percent of the total BW.

Measurement of liver fat content: Hepatic lipid content was determined by a Folch extraction and evaporation to dryness followed by a gravimetric measurement [24]. Pieces of liver were weighed and homogenized with 30 vol of chloroform: methanol (2:1). After standing overnight at room temperature, the homogenate was filtered, and a partition was performed in a separatory funnel by adding 0.2 vol of 0.05 N aqueous NaCl solution. When the two phases were completely separated, the lower fraction that contained the lipids dissolved in it was collected. Anhydrite CaCl2 was added to remove water vestiges and once again filtered to eliminate the salt. The filtered organic phase was dried in a rotavapor at 45°C. The residue was weighed, and the lipid content was expressed as weight/weight.

Histological evaluation: After the liver was removed, one piece was placed in 10% of formalin for histological evaluation. Liver histological examination by light microscopy was performed in a blinded manner. Hepatic tissue samples were fixed overnight, at room temperature in a 10% formalin buffer, pH 7 with 0.1 M phosphate-buffered saline solution. Samples were dehydrated in ethanol, embedded in paraffin wax, and

cut with a microtome. The resulting 5 micro-sections were stained with hematoxylin and eosin reagent for steatosis evaluation. The steatosis pattern was expressed with the presence of macro and microvesicular fat deposits and portal inflammation [25-26]. The histological features were assessed under low magnification (100x and 200x, respectively).

Biochemical determinations: Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were quantified by the UV-Kinetic (IFCC) method (Autopack, Wiener Diagnostics) in a CB350i autoanalyzer (Wiener Lab Rosario, Argentina).

Total serum cholesterol (T-C; mg/dL of serum), high-density lipoprotein-cholesterol (HDL-C; mg/dL of serum), and triglycerides (TG; mg/dL of serum) were determined by standardized methods (Roche Diagnostics GmbH, Mannheim, Germany) in a Hitachi 917 autoanalyzer (Hitachi, Tokyo, Japan).

Serum non-HDL cholesterol (non-HDL-C mg/dL), a set of atherogenic lipoproteins rich in apo B, was calculated as the difference between the T-C and HDL-C, given the loss of sensitivity of this parameter, which is usually significantly lower than serum HDL-C levels in rats as compared to humans [27].

Determination of oxidative stress indicators: Liver samples from each rat were homogenized (1:5 w/v) in a 100 mM Tris–HCl buffer, pH 7.75 with 2 mM EDTA and 5 mM $MgCl_2$ buffer [28]. Measurements were conducted according to Amado *et al.* [29] and Viarengo *et al.* [30] with modifications. Briefly, the homogenates were centrifuged at 4°C for 20 min at 10,000g and the supernatants were used for the assay. The reaction was followed in a 30 mM HEPES buffer, pH 7.2 with 200 mM KCl and 1 mM $MgCl_2$. The fluorescent probe 2',7' dichlorofluorescein diacetate (DCFH-DA) was added to the buffer at a final concentration of 40 µM. Then, after the addition of 5 to 15 µl of the sample, the reaction mixture was incubated at 35°C for 15 min. The fluorescent compound DCF, generated by the radical-dependent oxidation of the probe, was detected spectrofluorometrically at λ_{exc} = 488 nm and λ_{em} = 525 nm (F-3010 Hitachi). The results were expressed as arbitrary units/min mg of protein (AU/min mg prot). Protein content was calculated according to Lowry *et al.* [31] to standardize the quality of the sample.

The presence of different reactive species was tested by adding the enzymes superoxide dismutase (SOD, 300 U/ml) and catalase (CAT, 500 U/ml), dimethylsulfoxide (DMSO, 50 mM), glutathione (GSH, 5 mM), and deferoxamine (DF, 50 µM). Liver homogenates were incubated with DCFH-DA in the presence of these different scavengers for 15 min at 35°C. Then, the oxidation rate of the probe was performed as previously described.

Statistical analyses: To determine the sample size, a program provided by Harvard University (USA) was used (software available at: http://hedwig.mgh.harvard.edu/sample_size/size.html). The sample size for two groups was calculated using input parameters as follows: α error probability of 0·05, power probability of 0·08, and 50 units difference between the means based on previously determined serum T-C levels (60 ±10 mg/dl). As a result, a total sample size of > 6 rats per group was obtained. The results were expressed as mean values with their standard deviations (SD).

One-way analysis of variance (ANOVA) was used to compare data among groups. When a statistically significant difference was encountered, a Student-Newman-Keul's test was performed. Liver oxidative stress was presented as a percentage of inhibition of each scavenger compared to its basal group. Differences to the basal group were determined with Dunnett's multiple comparisons test. Histological information was analyzed using Chi^2 test. In all analyses, Bartlett's test for homogeneous variances was performed. Significance was set at the p <0.05 level.

The Statistical Product and Service Solutions for Windows 23 (SPSS, Inc., Chicago, IL) were used for the statistical analyses.

RESULTS

BW gain, visceral fat, and food intake: The BW gains throughout the experimental period, as well as the daily caloric intake of the three groups, are shown in Table **2**. At the beginning of the study, the mean body weight was 200 ±20g for all groups. After 5 weeks of feeding, BW gain was significantly higher in the AD group as compared with C and AD+Cur groups (*P* <0.05). The BW gain of the AD+Cur group was similar to the C group (*P* >0.05). Rats fed a C diet or AD (containing only AD or AD+Cur) did not show significant differences in energy consumption, despite some differences in energy content, fat content, and saturated fatty acids among the diets. Despite the similar consumption of the diets, supplementing the AD diet with Cur attenuated the BW gain significantly in AD+Cur group.

Table 2. Body and food consumption parameters and liver index in rats

Parameters	C	AD	AD+Cur	*P*
BW gain*(g)	81 ± 11a	113 ±12b	88± 21a	<0.05
Daily calorie intake (Kcal/100 g rat/day)	27 ± 2a	30 ± 3a	27± 4a	>0.05
HI (g/100 g BW)	4.0 ± 0.3a	5.9 ± 0.2 b	6.1 ± 0.6b	<0.001

C: control group (standard diet); AD: atherogenic diet group (saturated fatty acids and cholesterol diet); AD+Cur: atherogenic diet and curcumin group (saturated fatty acids, cholesterol diet, and oral curcumin). *BW: body weight gain throughout the experimental period. HI: means hepatic index (liver weight/total BW x 100). Different letters indicate statistically significant differences between groups. One-way ANOVA followed by SNK post hoc was used. Data are expressed as the mean ± standard deviation of the mean.

Table 3. Effect of administration of Cur on the distribution of visceral fat in AD-fed condition

Visceral fat	C	AD	AD+Cur	*P*
Perirenal (g/100 g BW)	1.1 ± 0.3a	1.8 ± 0.6b	1.0 ± 0.4a	<0.05
Mesenteric (g/100 g BW)	0.26 ± 0.05a	0.34 ± 0.08b	0.21 ± 0.03a	<0.01
Pelvic (g/100 g BW)	1.5 ± 0.4a	2.2 ± 0.6b	1.3 ± 0.4a	<0.05
Total visceral fat mass (g/100 g BW)	2.9 ± 0.8a	4 ± 1b	2.5 ± 0.8a	<0.05

Visceral fat weight per 100 g body weight (BW) of rats. C: control group (standard diet); AD: atherogenic diet group (saturated fatty acids and cholesterol diet); AD+Cur: atherogenic diet and curcumin group (saturated fatty acids, cholesterol diet, and oral curcumin). Different letters indicate statistically significant differences between groups. ANOVA+ Student-Newman-Keul's test (mean ± SD).

Total visceral fat content and distribution are shown in Table 3. The percentage of total visceral fat and distribution differed among the rats fed with the different diets. Rats consuming an AD diet showed the highest total visceral fat content as compared to C and AD+Cur groups (*P* <0.05). Furthermore, the percentage of perirenal, mesenteric, and pelvic fat showed the highest values for the AD group; the differences were significant as compared with other groups. Total visceral fat content, t as well as visceral fat distribution, remained similar within the C and the AD+Cur groups (*P* >0.05). Cur supplementation in the AD+Cur group significantly reduced the visceral fat; overall, the differential distribution of fat was also affected by the Cur intake.

Serum lipid profile: The serum lipids of the three groups of rats are shown in Figure 1. The rats fed the AD diet (AD or AD+Cur) showed the highest serum T-C (P <0.0001) and non-HDL-C (P <0.0001) levels; whereas AD, AD+Cur, and C groups attained similar serum concentrations of TG and HDL-C (P =0.54 and P =0.81, respectively). Cur supplementation did not induce changes in serum lipid profile when compared to the AD group.

Liver function, histopathology, and oxidative stress: The effects of Cur supplementation on liver morphology, fat content, enzymes and histology are presented in Figures 2 and 3. Hepatomegaly was induced by consuming the AD. The liver morphologic evaluation indicated qualitative differences among the three groups (Figure 2A). In the AD group, livers were grossly enlarged and became dull pale, slightly soft, friable to the touch and swollen; findings that were compatible with findings consistent with severe fatty liver-like disease; whereas those from rats fed AD+Cur were between sharp red and dull pale. Meanwhile, in group C, livers were sharp red and characterized by being soft, flexible, and small in volume.

AD diet induced hepatic steatosis in rats fed AD or AD+Cur as compared to those fed the C diet (P < 0.05; Figure 2B). In fact, the higher liver fat content in rats fed AD or AD+Cur was consistent with the HI (P < 0.001; Table 2).

The increased serum activities of AST and ALT enzymes (P < 0.0001; Figure 2C, D) denoted liver damage. Rats fed with a high fat and high cholesterol diet provoked the highest levels of both enzymes (P <0.0001); in contrast, the AD+Cur group achieved the lowest levels of ALT (P <0.0001) and a significant reduction of AST levels (P < 0.01) in comparison to those of group C.

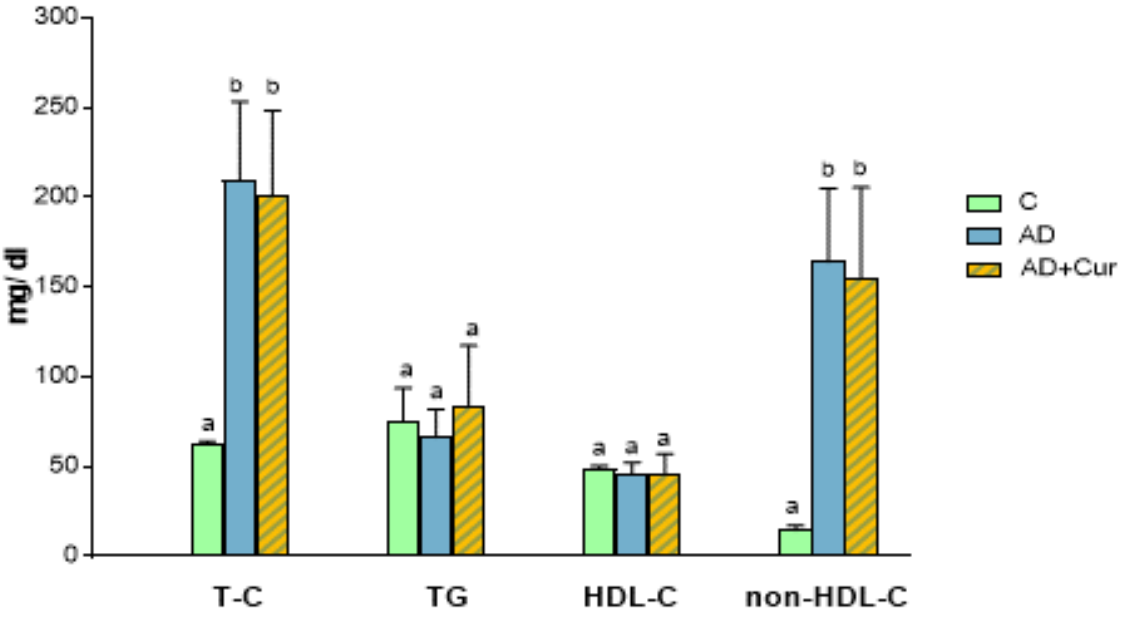


Figure 1. Supplementation with curcumin did not induce changes in serum lipid profile when compared to rats fed an atherogenic diet. Rats were assigned into three groups: C, control group, was given access to a standard diet; AD group, fed with atherogenic diet (saturated fatty acids and cholesterol diet) and AD+Cur group (atherogenic diet and curcumin, saturated fatty acids, and cholesterol diet plus oral curcumin). These diets were assigned for five weeks. Serum Total cholesterol (T-C), Triglycerides (TG), High-density lipoprotein (HDL-C), and non-HDL-C (non-HDL-C) were determined. Data are mean values, with standard deviations. Data were analyzed by using ANOVA and Student-Newman-Keul's test (P <0.05).

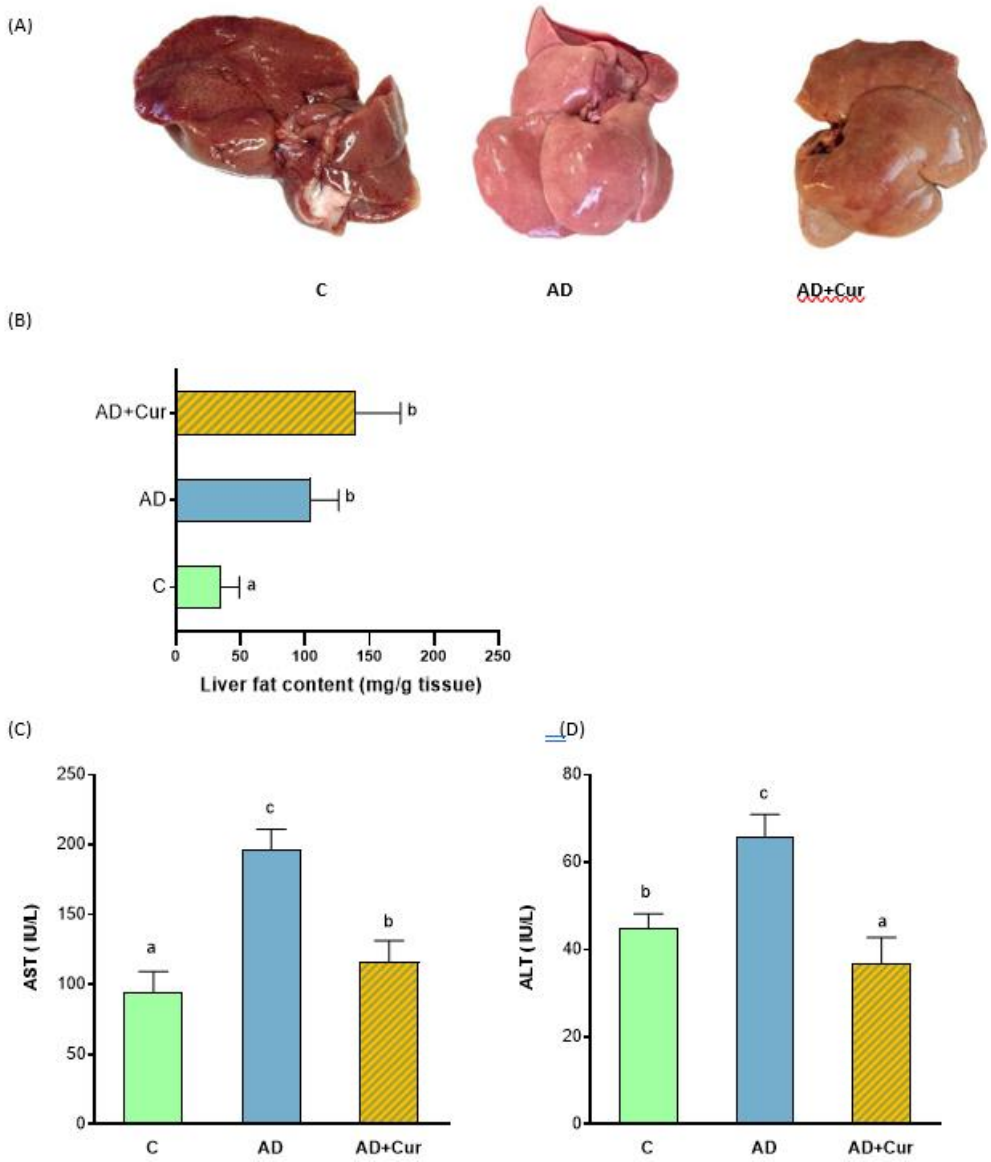


Figure 2. Supplementation with curcumin induces changes on hepatic morphology, liver fat content and enzymes of rats fed with an atherogenic diet. Rats were assigned into three groups: C, control group, was given access to a standard diet; AD group fed with atherogenic diet (saturated fatty acids and cholesterol diet) and AD+Cur group (atherogenic diet and curcumin, saturated fatty acids and cholesterol diet plus oral curcumin). These diets were assigned for five weeks. Livers were removed and (A) hepatic morphology, (B) liver fat content (mg/g tissue), (C) aspartate aminotransferase (AST) activity and (D) alanine aminotransferase (ALT) activity were determined. (A): AD induced hepatomegaly but morphologic differences among the three groups. Rats fed AD+Cur showed a liver between sharp red and dull pale. (B): hepatic steatosis was observed in rats fed AD or AD+Cur. (C) and (D): Cur induced a significant reduction in AST and ALT enzyme activity, respectively, provoked by AD diets. Data are mean values, with standard deviations. Data were analyzed by using ANOVA and Student-Newman-Keul's test (P <0.05). Mean values with different letters indicate statistically significant differences between groups.

The histopathological evaluation of the liver sections performed after hematoxylin-eosin staining showed remarkable differences among groups (Figure 3). Feeding rats with AD induced a higher steatosis and degeneration of the hepatocytes and the sinusoidal architecture than it did to those of the group C, fed a standard diet (Figure 3). In terms of hepatic steatosis, AD-fed rats induced grade 3 lesions with macrovesicular vacuoles while C rats were grade 0 (negative) for hepatic steatosis (Figure 3A). The marked increase in fat content displaced the nuclei of the affected cells to the periphery and induced microvesicular vacuoles and portal

inflammation (mononuclear cells were typically predominant), and this condition affected the whole organ (Figure 3B). Although the supplementation with Cur did not show a decrease in liver fat content, histopathological differences were evident. Even though hepatocytes exhibited steatosis grade 3 and microvesicular vacuoles, an apparent amelioration of inflammation was evident. The nuclei of these cells were not peripherally displaced in consistency with the absence of macrovesicular vacuoles. Then, Cur showed a hepatoprotective effect against inflammation (P =0.002, Chi^2 test) and fewer lipid depositions (Figure 3C).

The DCFH-DA oxidation rate was measured in the liver isolated from C and the animals treated with AD. The DCFH-DA oxidation was tested at different incubation times and it resulted to be linear with the experimental time, up to 15 min with high regression indexes under the conditions of the assay (Figure 4 inset).

In the homogenates from livers of the AD supplemented animals, a significantly higher DCFH-DA oxidation rate was observed, as compared to C homogenates (Figure 4, 2.2-fold). However, in the homogenates from livers of rats that received AD+Cur, the values were significantly reduced by 25% comparing them with the AD treated animals with Cur absence (Figure 4).

It is currently accepted that the DCFH-DA oxidation rate is dependent on a wide spectra of cellular oxidants [32]. The supplementation of the incubation medium with CAT (scavenger of H_2O_2), SOD (scavenger of O_2^-), DMSO (scavenger of $^{\bullet}OH$); GSH (general antioxidant) and deferoxamine (DF) (Fe chelator) were tested. The CAT, SOD and GSH addition to the assay conditions in C liver homogenates produced significant reductions of the oxidation rate of the dye. However, DMSO and DF showed no differences over the basal results (Table 4). Under the experimental conditions used here, when rats were fed with AD, CAT addition to the assay showed the highest inhibition of reactive species production. On the other hand, the homogenates of livers of animals receiving an AD+Cur showed a different profile of the effects of the scavengers supplemented to the reaction medium. A significant inhibition of the DCFH-DA oxidation rate by either DF or SOD was determined with no significant changes by the supplementation with CAT, GSH or DMSO.

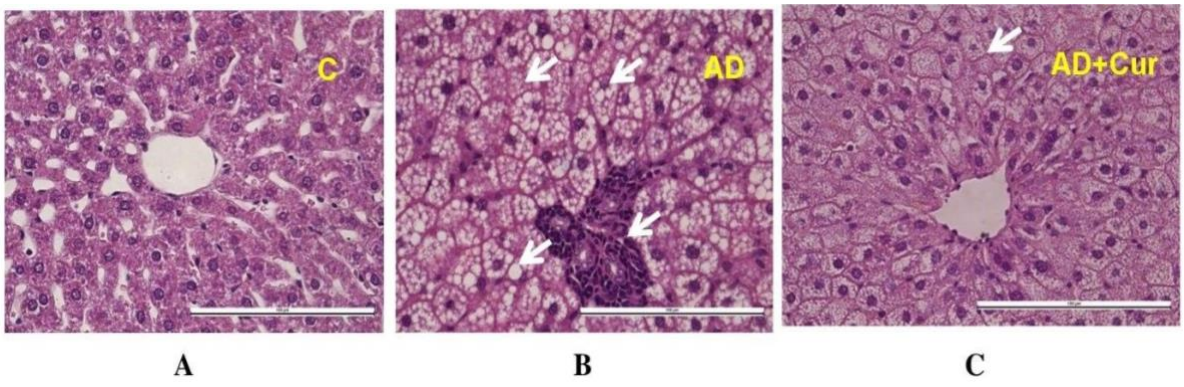


Figure 3. Supplementation AD with curcumin diminished the hepatic lipid deposition. Rats were assigned into three groups: C, control group, was given access to a standard diet; AD group fed with atherogenic diet (saturated fatty acids and cholesterol diet) and AD+Cur group (atherogenic diet and curcumin, saturated fatty acids and cholesterol diet plus oral curcumin). These diets were assigned for five weeks. Livers were removed for histological evaluation. (A) Liver of C group showing normal hepatocytes and sinusoidal architecture; (B) Liver of AD showing steatosis grade 3 with macrovesicular and microvesicular steatosis with portal inflammation, and the nuclei were peripherally displaced (arrows); (C) Liver of AD+Cur showing steatosis grade 3 with microvesicular steatosis with no inflammation. Cur shows a protective effect against inflammation. Samples stained with hematoxylin and eosin, 40x.

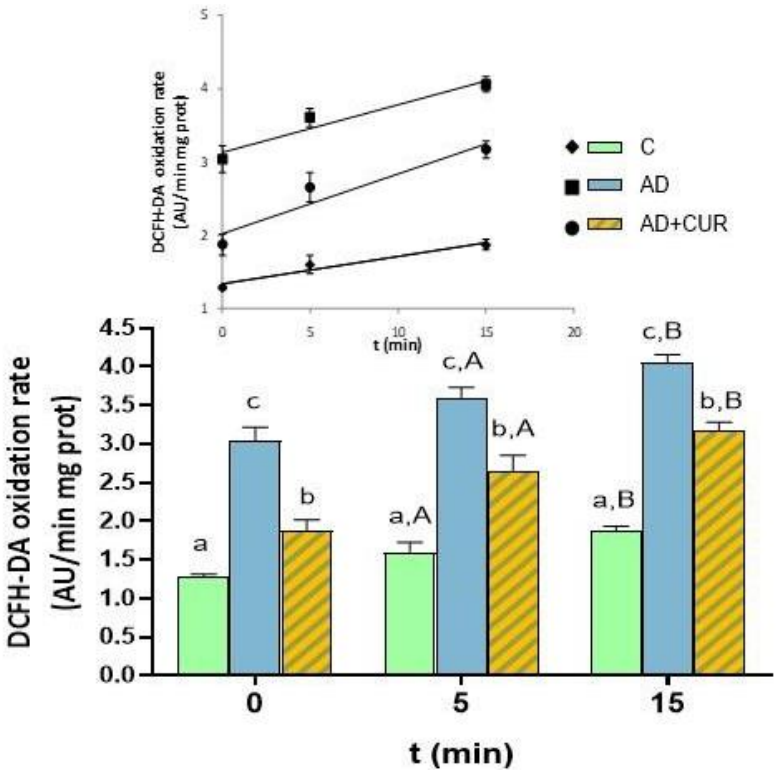


Figure 4. Supplementation with curcumin prevents hepatic oxidative stress of rats fed with an atherogenic diet. Rats were assigned into three groups: C, control group, was given access to a standard diet; AD group fed with atherogenic diet (saturated fatty acids and cholesterol diet) and AD+Cur group (atherogenic diet and curcumin, saturated fatty acids and cholesterol diet plus oral curcumin). These diets were assigned for five weeks. Livers were removed for oxidative stress assays. AD+Cur showed significant inhibition of the 2',7' dichlorofluorescein diacetate (DCFH-DA) oxidation rate by either deferoxamine (DF) or superoxide dismutase (SOD) with no significant changes on catalase (CAT), glutathione (GSH), or dimethyl sulfoxide (DMSO). **Inset**: linear regression curves for the reactive species generation during the DCFH-DA incubation period were for C y = 0.04x + 1.34 (R2 = 0.95); for AD y = 0.06x + 3.13 (R2 = 0.94); and for AD+Cur y = 0.08x + 2.02 (R2 = 0.91). a,b,c Different letters indicate statistically significant differences between groups; ANOVA, (mean ± SD, p <0.0001). A, B. Different letters indicate statistically significant differences between the incubation time; ANOVA, (p <0.001).

Table 4. Effect of the exposure to scavengers on the DCFH-DA oxidation rate by liver homogenates from different rat diets. Data are expressed as AU/minmg prot.

	C	AD	AD+Cur
BASAL	**1.87± 0.07a**	**4.1 ± 0.1c**	**3.2 ± 0.1b**
CAT	**0.8 ± 0.1A. a (55%)**	**0.8 ± 0.1 A. a(80%)**	**3.0 ± 0.1b (6%)**
SOD	**1.56 ± 0.09A. a (17%)**	**3.6 ± 0.1A. c (11%)**	**2.5 ± 0.3A. b(20%)**
DMSO	**1.77 ± 0.05a (5%)**	**3.4 ± 0.2A. c(17%)**	**3.0 ± 0.1b (7%)**
GSH	**1.56 ± 0.06A. a (16%)**	**2.90 ± 0.09A. b (28%)**	**3.09 ± 0.03c (3%)**
DF	**1.9 ± 0.1a (0%)**	**2.7 ± 0.3A. b(33%)**	**1.5 ± 0.2A. a(53%)**

C: control group (standard diet); AD: atherogenic diet group (saturated fatty acids and cholesterol diet); AD+Cur: atherogenic diet and curcumin group (saturated fatty acids and cholesterol diet and oral curcumin). CAT: catalase; SOD: superoxide dismutase; DMSO: dimethylsulfoxide; GSH: glutathione; DF: deferoxamine. Percentage between brackets indicates the % of inhibition of each scavenger compared to their basal group. Different letters indicate statistically significant differences between groups within each scavenger. ANOVA + Student-Newman-Keul's test (p<0.001). [A] Significantly different to their own basal group. ANOVA + Dunnett's multiple comparisons test (p<0.001).

DISCUSSION

The present study demonstrated that supplementation with Cur to a high-fat high-cholesterol diet has a promising protective role acting as an anti-obesity agent and preventing liver injury; however, its cardioprotective effect could not be proved.

The consumption of a diet high in fat and cholesterol is associated with the development of certain metabolic disorders that promote chronic diseases.

To evaluate the benefits of Cur supplementation in preventing hepatic and metabolic alterations, the present study was performed in an experimental rat model of dietary induced hypercholesterolemia. This model appears to be more suitable for assessing liver alterations than other existing animal models, which require genetic deficiencies. Our results showed the capacity of oral Cur to reduce the total BW gain and body fat deposition, after 5 weeks of treatment. The lower BW gain observed could be attributed to Cur's increase of basal metabolism and energy expenditure [33]; further studies will be designed to elucidate these findings. Besides, Kim *et al.* [34] reported a significant decrease in BW gain and adiposity in rats fed a diet containing 10% of *turmeric* powder for 6 weeks. In concordance, as an anti-obesity agent, Cur lowers body fat and reduces weight [35]. Indeed, Cur was reported to down-regulate the expression of genes involved in energy metabolism and lipid accumulation by decreasing the level of intracellular lipids [36]. In the adipose tissue, Cur suppresses the angiogenesis necessary for tissue growth [36]. In addition, Cur improves inflammation associated with obesity and may offer a cardioprotective effect for preventing hypercholesterolemia-induced atherosclerosis [37].

In the present study, the highest increase in the amount of visceral fat was detected in rats fed with the AD, being this one of the risk factors for CVD. Nevertheless, the main predictors for CVD (non-HDL, HDL and total cholesterol) were not affected by Cur treatment. Meanwhile, Cur supplementation ameliorated some consequences of consuming a high-fat high-cholesterol diet. However, the percentage of liver fat mass could not be reduced in Cur-treated rats. Five weeks exposition to Cur was not sufficient to improve the effects of hypercholesterolemia in the liver, highly associated with this detrimental AD. In fact, Cur proved to be a promising tool for improving liver function. However, three times higher values of hypercholesterolemia than normal prevented Cur from showing its beneficial effect on hepatic steatosis. These results may serve as a note of caution in the use of this type of diet. Further long-term studies including high fat and mild cholesterol diet should be performed.

As it was observed, the high-fat high-cholesterol feeding caused alterations in the lipid homeostasis evidenced by the increased T-C and non-HDL-C levels; but there was a lack of a lowering effect on serum T-C and non-HDL- C levels by Cur supplementation. Although hyperlipidemia was reported as one of the major risk factors for fatty liver [38], the serum TG levels were similar among groups. This may suggest that serum TG levels may not be an accurate indicator to evaluate fatty liver disorders in animals and, probably, in humans. It could be assumed that the hepatomegaly found in the AD groups was possible due to the poor management of the dietary cholesterol metabolism [39]. Supplementation with Cur to AD did not show an improvement in the lipid profile. The incapacity to normalize the circulating levels T-C, non-HDL-C, and TG was not due to the low bioavailability of this compound, since Cur was co-administered in combination with an absorption enhancing adjuvant (piperine) [40]. However, plasma levels of Cur were not determined; the lack of this data might be a limitation that should be considered in further studies. These results are consistent with some meta-

analyses which have found that Cur supplementation has apparently no effect on serum T-C, LDL-C, TG and HDL-C levels when considering heterogeneous populations [41]. Therefore, the proposed cardiovascular protective effects of Cur could be attributed to other mechanisms than lipid-lowering and HDL-C enhancing activities. Mechanisms such as mitigation of lipid peroxidation, platelet aggregation, endothelial dysfunction, and inflammation among others [41]. The results obtained are likely to be attributed to the trial duration, but not to an insufficient dose. According to the above, and taking into account that the metabolic rate in rats is higher than in humans, the amount of *turmeric* used for rats in the present study could be translated to an equivalent human dose of about 1 g of Cur per day, assuming that the average mass of a human is about 70 kg [42]. This amount of *turmeric* consumption is rational considering the capsules commercially available.

In contrast, other studies reported hypocholesterolemic effects of Cur in rats. Different mechanisms have been proposed for those hypolipidemic actions, including inhibition of intestinal cholesterol absorption, inhibition of hepatic lipid biosynthesis, stimulation of bile secretion and modulation of the expression and/or activity of lipoprotein receptors [40]. Rats fed with a 45% high-fat diet with or without Cur (0.1% w/w) for 8 weeks showed significantly decreased serum TG, T-C and LDL-C levels in comparison with controls. It was then suggested that Cur regulates the expression of genes involved in cholesterol homeostasis [43]. Even more, Cur was reported to transcriptionally inhibit 3-hydroxy-3-methylglutaryl-coenzyme; a reductase (HMG-CoA) activity in a 18 week atherogenic model [44]. Some of the lipid-lowering effects of Cur in plasma might be responsible for its beneficial effects against several diseases in which hyperlipidemia plays important roles, such as inflammation, diabetes, obesity, and atherosclerosis [45].

In the present study, the AD induced histopathological changes in the liver with an intense inflammatory infiltrate and steatosis which was observed by hematoxylin and eosin technique. In AD and AD+Cur, some other histological techniques could be used to differentiate the steatosis grading more accurately; then, this limitation should be considered. Being the liver a major site for the synthesis, oxidation, metabolism, storage and distribution of lipids playing an essential role in regulating energy metabolism [46], the chronic consumption of this type of diets may lead to serious metabolic and liver alterations, such as fibrosis and cirrhosis [47]. Moreover, the additive cholesterol accretion in the liver brought steatohepatitis due to cholesterol-induced toxicity [48]. In the present experiments, Cur supplementation significantly decreased visceral fat and attenuated liver histopathology. The increase in the hepatic content of lipid was an important feature and showed the disturbance of lipid homeostasis due to a high-fat high-cholesterol diet. A crucial fact is that even though the total amount of fat in the liver remained similar in rats fed with AD or AD+Cur, it remains unclear how the contribution of each component -TG and cholesterol- affected the results. The morphological studies revealed a large difference in the hepatic appearance of each group of rats. Indeed, the AD+Cur rats evidenced the existence of a different liver fat composition.

Hepatic enzymes such as ALT and AST are often used as markers of hepatic function and their serum increase is used as a marker of ongoing liver damage [49]. The results of the present study showed a significant increase in the serum activities of the AST and ALT in all rats consuming AD. The use of Cur evidenced its capacity to reduce AST and ALT as well as liver damage. These findings, confirmed by the histopathological observations of the liver, revealed that Cur could prevent liver damage and toxicity. The use of Cur evidenced its capacity to

reduce AST and ALT as well as liver damage. Even though, specific studies were not performed to evaluate the correlation between oxidative stress development and the increase in these serum enzymatic activities in rats receiving the AD diet, the measured enhancements on cellular radical generation lead to an increase in lipid peroxidation that favored the release of the liver enzymes to the blood. Thus Cur, by decreasing the oxidative stress, was able to prevent the leakage of the enzymes.

Feeding rats an AD induced hepatic steatosis due to a long-term accumulation of TG in the hepatocytes, often accompanied by increased oxidative stress. The excessive production of ROS and the antioxidant imbalance can provoke an acute inflammatory response, leading to progressive liver damage. ROS contribute to ischemia/regeneration, necrosis and apoptosis [14]. In this study, the oxidation rate of DCFH-DA was significantly increased in AD rats as compared to C, suggesting that a high-fat high-cholesterol diet contributed to a cellular over production of total reactive species. H_2O_2, diffuses freely into the tissue increasing the oxidative stress and causes further oxidative damage. H_2O_2 is especially toxic through the Fenton reaction with Fe^{2+}, where it becomes extremely reactive to $^{\bullet}OH$, which causes severe damage to membranes, proteins, and DNA. Moreover, the excessive ROS production could overwhelm the scavengers leading to necrosis and apoptosis of hepatocytes [50]. Cur supplementation attenuated the oxidation rate of the dye induced by the AD, showing its role in the management and protection of oxidative damage associated with liver diseases. Cur was described to exert some hepatoprotective activity by preventing liver toxicity and reducing the activity of ALT and AST enzymes. These results were supported by the histopathological findings of the liver, observing an improvement in the morphological features of the hepatic tissue.

As shown here, when liver homogenates were exposed to scavengers, rats fed AD, showed the highest inhibition for CAT and were followed by DF, related to a H_2O_2 and $^{\bullet}OH$ production, respectively. In contrast, the Cur supplemented diet reduced the appearance of several reactive species but provoked the significant production of $^{\bullet}OH$. The lowest inhibition for CAT found in rats fed AD+Cur could be caused by a lower production of H_2O_2 in the presence of Cur that prevented oxidative stress. Farzaei *et al.* [13] showed that Cur inhibits lipid peroxidation and neutralizes ROS.

CONCLUSION

Since findings suggest that oral supplementation with Cur improves some of the metabolic alterations associated with the consumption of an AD, Cur would act as an anti-obesity agent reducing body fat and weight. It is evidenced here that Cur could not improve hypercholesterolemia and hepatic steatosis caused by DA; possibly due to the high concentration of cholesterol administered in the diet. Certainly, the amount of lipids supplied and the endogenous synthesis could exceed the hepatocyte's ability to manage its turnover rate thus establishing steatosis. However, the histological findings and the capacity of Cur to prevent liver inflammation in addition to its antioxidant effect, were evidenced in the reduction of ROS detected by the DCFH-DA assay. Lastly, the valuable use of Cur as a dietary supplement has a promising protective role against liver diseases although its cardioprotective capacity remains unproved. This study provides supporting evidence to confirm the beneficial effects of curcumin from the point of view of functional food science.

List of Abbreviations: AD: atherogenic diet, ALT: alanine aminotransferase, AST: aspartate aminotransferase, BW: body weight, C: Controls, Cur: curcumin, CAT: catalase, CVD: cardiovascular disease, DCFH-DA: dichlorofluorescein diacetate, DF: deferoxamine, DMSO: dimethylsulfoxide, DRCD: diet-related chronic diseases, GSH: glutathione, HDL-C: high-density lipoprotein-cholesterol, HI: hepatic index, NAFLD: non-alcoholic fatty

liver disease, non-HDL-C: non-HDL cholesterol, ROS: reactive oxygen species, SOD: superoxide dismutase, T-C: Total serum cholesterol, TG: triglycerides.

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Authors' contributions: MAE carried out the investigation, acquisition of data, analysis and interpretation of data. PG helped design the study, analyzed the data and wrote the manuscript. CR, JC and CO processed samples, performed the lab determinations and analyzed the data. TS helped design the study, conducted the study, and analyzed the data. CM analyzed the histopathological data. VZ analyzed the serum lipids. SP designed the study, supervised the development of the study, analyzed the data, and wrote the manuscript. SMF analyzed the data and was a major contribution in manuscript preparation. EVM designed the study, supervised the development of the study, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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