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# Kinkan orange protects hypercholesterolemic rats against dyslipidemia and oxidative stress

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**Abstract:** We investigated the effect of dietary supplementation with kinkan orange on growth, adiposity, metabolic parameters, and oxidative stress in rats with diet-induced hypercholesterolemia. Female Wistar rats (6-8 weeks) were fed a AIN-93M diet (Control); AIN-93M diet containing 5% kinkan orange (CTkinkan); Hypercholesterolemic diet, containing 1% cholesterol and 25% fat (Hyper); or Hypercholesterolemic diet containing 5% kinkan orange (Hyperkinkan). Hypercholesterolemic diet increased body weight, adiposity, serum alanine transaminase (ALT), creatinine, cholesterol and triglycerides, hepatic total lipids, cholesterol, and triglycerides, and hepatic oxidative stress. Supplementation with kinkan reduced the serum and hepatic lipid content, decreased serum ALT, besides improving the antioxidant status in liver tissue of hypercholesterolemic animals. Moreover, HDL-cholesterol increased in both groups supplemented with kinkan orange (CTkinkan and Hyperkinkan). Our data suggest that diet supplementation with kinkan orange may consist of a valid strategy to prevent or reduce dyslipidemia and oxidative stress in hypercholesterolemic rats.

**Key words:** citrus, dyslipidemia, high-fat diet, oxidative stress.

## INTRODUCTION

Hypercholesterolemia is the major risk factor for cardiovascular diseases (CVD) that enhance reactive oxygen species (ROS) generation in blood and tissues (Baldissera et al. 2017, El-Tantawy 2015).

Nutritional intervention can be one of the major strategies to prevent CVD. Studies suggest that citrus fruits intake is associated with prevention of CVD development, for promoting reduction in serum levels of cholesterol and triglycerides (Zheng et al. 2017). The health benefits of citrus fruits can be attributed to their high amounts of phytochemicals and bioactive compounds (Toth et al. 2016, Zou et al. 2016). Among the various possible mechanisms, regulation of lipid metabolism, reduction of oxidative stress and attenuation of inflammation are reported (Zou et al. 2016, Zhao et al. 2017, Zheng et al. 2017).

Previous studies have associated orange intake with beneficial effects on lipid metabolism and oxidative stress. Deyhim et al. (2007) have shown that 2.5, 5.0 or 10% orange pulp intake increased antioxidant plasma levels, hepatic antioxidant enzymes activity (superoxide dismutase and catalase) and improved the lipid profile in orchidectomized rats. Another study showed that orange juice consumption provided a protection of mononuclear blood cells against oxidative DNA damage in healthy subject (Guarnieri et al. 2007). Giglio et al. (2016) related that *Citrus bergamia Risso* (family Rutaceae), protects against free-radical damage, raises high density lipoprotein cholesterol (HDL-C),

and reduces triglycerides accumulation in the liver, in studies involving animals and humans.

Kinkan orange (*Fortunella japonica*), originally from China, is a source of dietary fiber and bioactive compounds, including ascorbic acid, terpenoids, carotenoids, flavonoids, and essential oils (Choi 2005, Lim 2012, Sadek et al. 2009). A previous study of our group showed that kinkan orange has chemical characteristics similar to those found in Brazilian citrus fruits, such as pH (4.22±0.03), soluble solids (21.1± 0.10 ºBrix), titratable acidity (1.14±0.16 g/100ml), vitamin C (86.45±6.65 mg/100ml), total phenolic contents (0.09 ± 0.0010 mg/g), and antioxidant capacity (52.99 ± 8.83 %) (Oliveira & Diniz 2015). Kinkan orange is eaten whole as its rind is extremely sweet, fragrant and pleasant. It can also be processed into candies, preserves, marmalade, and jelly, or it can be sliced and added to salads (Choi 2005).

It is believed that kinkan orange consumption may protect against cardiovascular risk factors, such as dyslipidemia and oxidative stress. Therefore, the objective of this study was to evaluate the effect of kinkan orange consumption on weight parameters, lipid profile, oxidative stress and adiposity in rats receiving control or hyperlipidemic diet.

## MATERIALS AND METHODS

### Experimental Design

Forty female Wistar rats aged from 6-8 weeks were randomly distributed according to their weight into four groups of 10 animals to receive for 4 weeks AIN-93M diet (calorie density 3.95 kcal/g - Control group); AIN-93M diet containing 5% kinkan orange (calorie density 3.98 kcal/g - CTkinkan group); Hypercholesterolemic diet, containing 1% cholesterol and 25% fat (calorie density 4,81 kcal/g - Hyper group); or Hypercholesterolemic diet containing 5% kinkan orange (calorie density 4,84 kcal/g - Hyperkinkan group). Whole oranges excluding seeds were sliced, dried at 60 °C and ground before being added to the diets. Diets were offered *ad libitum* in pellet form.

The animals were maintained in controlled conditions of temperature (22 ± 3 °C) and light (12 h light/dark cycle), with free access to water and diet. After four weeks and 12 hours fasting, animals were anesthetized intraperitoneally with ketamine/xylazine (100 and 12 mg/kg body weight respectively), and blood collected from the axillary artery. Serum was separated by centrifugation (12 000*g*) for 10 minutes, adipose tissue (epididymal, peritoneal, and mesenteric), and liver tissue were collected. The experimental protocol was carried out according to the guidelines of the Ethics Committee on Animal Use (CEUA) of Federal University of Minas Gerais (protocol 069/10).

### Assessment of food, body weight, energy intake and adiposity

Food intake and body weight were recorded weekly. The energy intake was calculated as the product of food intake and calorie density. The relative weights of liver and the adiposity index were estimated as recommended (Paulino et al. 2008).

### Lipid profile, serum enzymes, urea and creatinine

#### *Serum triglycerides and total cholesterol*

All tests were performed by enzymatic colorimetric assay as recommended by the manufacturer's protocols (Labtest kit, Brazil), adapted for a microplate assay (Fazio et al. 1997). In brief, 10 l of serum was mixed with 100 ml of cholesterol reagent, and the absorbance at 492 nm was read (Molecular Devices microplate reader) after a 10-min incubation at 37 ° C.

### *Cholesterol carried by lipoproteins*

Serum lipoproteins fractioning was performed through gel filtration chromatography with a Fast Protein Liquid Chromatography (FPLC) system (Waters model 600), using a Superose column (Pharmacia) (Fazio et al. 1997).

Three samples from serum from each group (pooled serum of animals 1 2, 3 -sample 1; 4, 5, 6 – sample 2; 7, 8, 9 and 10 - sample 3) were used for lipoprotein determination. The pooled serum (a 100 µl aliquot) was loaded onto a Superose 6 column and separated at a flow rate of 0.5 ml/min, with a buffer containing 0.15 M NaCl, 0.01 M $Na_2HPO_4$, 0.1 mM EDTA, pH 7.5. Forty fractions were collected (0.5 ml each), with the lipoproteins (high density lipoprotein) being contained within tubes 17–37. Cholesterol levels in the fractions were determined as described for serum samples, except that 100 µl of each fraction were mixed with the cholesterol reagent (1:1 vol ratio).

### *Hepatic lipids*

Hepatic total lipids were extracted from liver using organic solvents (Folch et al. 1957). Briefly, 100 mg of liver was homogenized in a chloroform:methanol solution (2: 1). After adding methanol and centrifugation, the supernatant was transferred to a pre-weighed container and mixed with chloroform and 0.73% NaCl solution. After another centrifugation, the upper phase was discarded and washed three times with Folch's solution. The tubes were placed at 37 ° C and total liver fat was gravimetrically quantified. The lipid extracts were resuspended in 500 µL of isopropanol and the determination of hepatic cholesterol and triglyceride concentrations was performed using commercial kits (Labtest kit, Brazil).

### *Determination of serum transaminases activities, urea and creatinine*

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, and creatinine were determined using biochemical assay kits (Labtest, Brazil).

### Oxidative stress in the liver

Fragments of tissue (approximately 100 mg) were homogenized in 1mL (w/v) of 50mM ice cold phosphate buffer pH 7.03. After centrifugation at 12.000 rpm, 10 min, 4 ° C, supernatants were kept at -70 ° C for assays. The content of total liver protein was determined according to Lowry et al. (1951).

TBARS analysis was performed as previously described (Buege & Aust 1978). Briefly, 200 µL of supernatant were mixed with a solution containing thiobarbituric acid (TBA 0.375%) in acid solution (15% trichloroacetic acid and 0.25 M hydrochloric acid), incubated in boiling water (95 ° C) for 15 min and subsequently placed on ice for cooling. Samples were mixed with 600 µL of n-butanol and centrifuged at 6000 rpm (Neofuge 15R, Heal Force) for 10 min. Aliquots of the supernatant (150 µL) were transferred to microplate (96 wells) and the absorbance was read at 535 nm on a microplate reader (Biotek, ELx800 absorbance microplate reader, VT, USA). The results were expressed in µmol Malondialdehyde (MDA)/g protein.

Hepatic hydroperoxide concentration was determined by the ferrous oxidation of xylenol orange (Nourooz-Zadeh et al. 1994). Briefly, 20 µL of supernatant were transferred into the microplate, followed by the FOX-2 reagent. After 30 min at room temperature, the absorbance was measured spectrophotometrically at 560 nm. In order to reduce hydroperoxides with triphenylphosphine (TPP), 15 µL of supernatant and 5 µL of TPP solution in methanol (10 mM)

were added directly in the microplate and kept for 30 min at room temperature, followed by the FOX-2 reagent. After 30 min at room temperature, the absorbance was measured spectrophotometrically at 560nm. The hydroperoxide concentrations were calculated by the difference between the measurements without TPP from those with TPP. The results were normalized by the protein content and expressed as µmol hydroperoxides/g protein.

### *Antioxidant enzymes activities*

Superoxide dismutase (SOD) activity in the liver homogenates was determined based on the inhibition of pyrogallol autoxidation (Dieterich et al. 2000). Catalase activity was measured, considering the decrease in the absorbance at 240 nm (Nelson & Kiesow 1972). The results were expressed in enzyme units /g of liver proteins.

### Statistical analysis

In this study, completely randomized design was performed with four treatment groups. Data were analyzed statistically by the Shapiro-Wilk test to verify for normal distribution, and Grubbs' test was used to detect outliers. The effects of cholesterol in the diet (control or hypercholesterolemic) and the presence or not of kinkan orange supplementation (without or with kinkan) and the interaction between these investigated factors (cholesterol x kinkan) were assessed by two-way analysis of variance (ANOVA). When the two-way ANOVA detected significance for the interaction and the main effects, the differences were assessed with Bonferroni's multiple comparisons post hoc test at $P \leq 0.05$. Data were expressed as means ± S.E.M. The analysis was carried with Prism software 7.0 (GraphPad Software, USA).

## RESULTS

### Effect of kinkan orange on food intake and weight parameters

Hypercholesterolemic diets (Hyper and Hyperkinkan) showed higher caloric density than control diets (Control and CTkinkan), leading to increased body weight ($P < 0.0001$) and relative liver weight ($P < 0.0001$), supported by increased adiposity ($P = 0.0003$). On the other hand, dietary cholesterol did not influence *ad libitum* food nor energy intake. Kinkan orange supplementation did not change food, energy intake and weight parameters ($P > 0.05$) (Table I).

### Effect of kinkan orange on liver and renal function

Serum AST and urea were both reduced by kinkan administration (CTkinkan and Hyperkinkan groups, $P = 0.002$ and $P = 0.03$, respectively). For serum ALT levels, there was an interaction effect between experimental factors (cholesterol X kinkan, $P < 0.0001$). Thus, ALT levels were lower in animals fed hypercholesterolemic diet supplemented with kinkan orange than in the other groups (Hyperkinkan group, $P < 0.0001$). Serum creatinine levels were significantly increased by the hypercholesterolemic diet ($P < 0.0001$) and reduced by supplementation with kinkan orange (CTkinkan and Hyperkinkan groups, $P = 0.005$) (Table I).

### Changes in lipid profile

The serum and hepatic lipids were considerably increased by the hypercholesterolemic diet (Table I). For all these measured parameters, an interaction effect between cholesterol and kinkan supplementation was observed; thus, the concentration was higher in Hyper group related to the control groups (Control and CTkinkan). Interestingly, the hypercholesterolemic rats fed with kinkan orange (Hyperkinkan) showed a

**Table I. Food and energy intake, body weight, adiposity and metabolic parameters of animals fed with different diets.**

| | Experimental groups | | | | Two-way Anova *P value* | | |
|---|---|---|---|---|---|---|---|
| **Variables** | **Control** | **CTkinkan** | **Hyper** | **Hyperkinkan** | **Cholesterol** | **Kinkan** | **Cholesterol x Kinkan** |
| Food intake (g/animal/day) | 2.80 ± 0.21[a] | 2.78 ± 0.23[a] | 2.39 ± 0.31[a] | 2.80 ± 0.21[a] | 0.12 | 0.80 | 0.84 |
| Energy intake (kcal/animal/day) | 11.07 ± 0.83[a] | 11.09 ± 0.90[a] | 11.50 ± 1.50[a] | 10.93 ± 1.67[a] | 0.92 | 0.84 | 0.82 |
| Final body weight (g) | 222.8 ± 4.25[a] | 215.0 ± 4.04[a] | 252.5 ± 9.15[b] | 260.9 ± 2.80[b] | <0.0001 | 0.96 | 0.17 |
| Relative liver weight (%) | 3.27 ± 0.11[a] | 3.17 ± 0.11[a] | 5.21 ± 0.14[b] | 5.12 ± 0.15[b] | <0.0001 | 0.45 | 0.96 |
| Adiposity index (%) | 1.90 ± 0.13[a] | 1.90 ± 0.21[a] | 2.55 ± 0.13[b] | 2.60 ± 0.17[b] | 0.0003 | 0.89 | 0.84 |
| Serum | | | | | | | |
| AST (U/mL) | 39.32 ± 3.00[a] | 28.96 ± 1.73[b] | 39.52 ± 5.52[a] | 27.48 ± 1.46[b] | 0.85 | 0.002 | 0.80 |
| ALT (U/mL) | 15.26 ± 0.53[a] | 13.99 ± 0.80[a] | 19.73 ± 2.18[a] | 8.38 ± 0.21[b] | 0.59 | <0.0001 | <0.0001 |
| Urea (mmol/L) | 3.98 ± 0.26[a] | 3.25 ± 0.17[b] | 3.50 ± 0.13[a] | 3.38 ± 0.24[b] | 0.27 | 0.03 | 0.25 |
| Creatinin (mmol/L) | 37.84 ± 2.86[b] | 30.84 ± 2.75[a] | 51.40 ± 2.60[b] | 43.27 ± 1.63[c] | <0.0001 | 0.005 | 0.82 |
| Cholesterol (mg/dL) | 153.3 ± 14.45[a] | 146.6 ± 11.73[a] | 281.9 ± 26.97[b] | 157.6 ± 17.05[a] | 0.0007 | 0.001 | 0.004 |
| Triglycerides (mg/dL) | 115.5 ± 0.50[a] | 123.9 ± 4.92[a] | 159.6± 8.79[b] | 110.5 ± 7.44[a] | 0.03 | 0.005 | <0.0001 |
| Liver (mg/g tissue) | | | | | | | |
| Total lipids | 47.86 ± 4.14[a] | 39.50 ± 2.18[a] | 280.80 ± 12.67[b] | 233.10 ± 8.10[c] | <0.0001 | 0.002 | 0.022 |
| Cholesterol | 2.16± 0.14[a] | 1.91 ± 0.12[a] | 24.55 ± 1.56[b] | 19.90 ± 1.04[c] | <0.0001 | 0.015 | 0.03 |
| Triglycerides | 8.68 ± 1.20[a] | 1.84 ± 0.11[b] | 16.60 ± 0.66[c] | 12.76 ± 1.23[d] | <0.0001 | <0.0001 | 0.04 |

**Relative liver weight, liver weight/body weight×100; adiposity index, sum of the peritoneal, mesenteric and epididymal fat pads divided by the body weight; AST, aspartate transaminase; ALT, alanine transaminase. Values are expressed as mean ± S.E.M (n = 10 rats). Values not sharing a common superscript letter within a row are statistically significant (p<0.05) using two-way ANOVA followed by post hoc Bonferroni test.**

marked reduction in serum levels of cholesterol ($P$ = 0.001) and triglycerides ($P$ = 0.005), and in hepatic total lipids ($P$ = 0.002), cholesterol ($P$ = 0.015), and triglycerides ($P$ < 0.0001), compared to Hyper group. The FPLC cholesterol elution profiles (Fig. 1a) demonstrated that serum cholesterol was mainly carried by HDL in all groups. Control groups (Control and CTkinkan diets) showed similar lipoprotein profiles, while hypercholesterolemic group showed lower average content of cholesterol in lipoproteins. The supplementation with kinkan orange in hypercholesterolemic condition (Hyperkinkan diet) resulted in an increase of circulating HDL-cholesterol, improving the lipoprotein profile (Fig. 1b). Lipoprotein cholesterol concentration was affected by cholesterol ($P$ < 0.0001) and kinkan supplementation ($P$ < 0.0001) and an interaction effect between these dietary factors was also observed ($P$ = 0.0001).

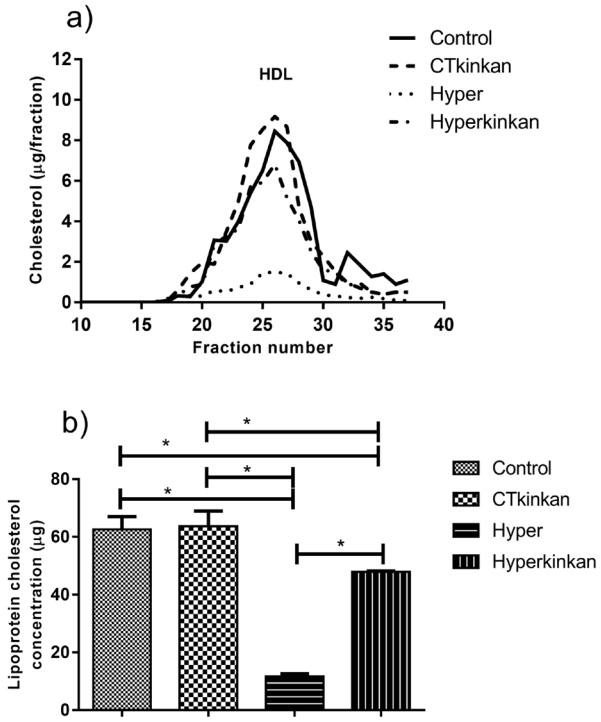


**Figure 1. Cholesterol distribution on circulating lipoproteins in rats given Control, CTkinkan, Hyper and Hyperkinkan diets, after 4 weeks of experiment. a) Cholesterol profile found in lipoprotein fractions analyzed. b) Mean values of the sum of the cholesterol concentration found in the different experimental groups. Gel filtration chromatography was performed using a Superose 6 column (Pharmacia) on a Waters 600 FPLC system. The pooled serum (a 100µl aliquot) was loaded onto a Superose 6 column and separated as described above. Cholesterol determinations were measured in the microplate assay. Data points represent mean values for cholesterol from three pooled serum from each group (n=5). *Bars in Figure 1b represent statistical significance, p < 0.05, using two-way ANOVA followed by post hoc Bonferroni test.**

### Changes in antioxidant capacity

Animals fed Control and CTkinkan diets had similar results of hepatic TBARS, hydroperoxides, SOD and catalase activities (Fig. 2a, 2b, 3a, and 3b). Hypercholesterolemic diet increased TBARS and hydroperoxides levels ($P < 0.0001$ and $P = 0.0002$, respectively) compared to animals fed control diets. Kinkan orange administration normalized hydroperoxides levels and increased catalase activity ($P = 0.036$ and $P = 0.0053$, respectively) in animals fed hypercholesterolemic diet (Hyperkinkan group).

## DISCUSSION

Hypercholesterolemia is well-known as one of the most important risk factors of atherosclerosis. Our data confirmed previous studies showing that diet containing 25% soybean oil and 1% cholesterol induces weight gain, dyslipidemia, hepatic lipid accumulation, and oxidative stress in female rats (Abreu et al. 2014, Silva et al. 2013). Many studies have indicated the consumption of fruits for prevention and treatment of cardiovascular diseases, paying special attention to the composition of fruits and the mechanisms of action (Deyhim et al. 2007, Zhao et al. 2017, Zheng et al. 2017). The flavonoid content of different Brazilian citrus varieties was reported to be found more abundantly in the fruit peels than in juices (Pereira et al. 2017). The nutrient composition of raw kinkan (*Fortunella* spp.) (exclude 7% seeds) per 100g edible portion was reported as: water 80.85 g, energy 71 kcal (296 kJ), protein 1.88 g, total lipid 0.86 g, ash 0.52 g, carbohydrate 15.90 g and dietary fiber 6.5 g (Lim 2012).

Because kinkan orange can be eaten whole and showed high content of dietary fiber, vitamin C, total phenolics and considerable antioxidant capacity compared to other Brazilian citrus fruits (Oliveira & Diniz 2015), we conducted the present study to investigate its effects on the metabolic changes in rats fed with control or hypercholesterolemic diet. We found that supplementation with 5% kinkan orange did not affect the caloric density of diets and reflected in the similar results of food and energy intake, body weight, liver relative weight and adiposity index in control groups (Table I).

Dietary-induced hypercholesterolemia is a strategy used to study lipid metabolism. In

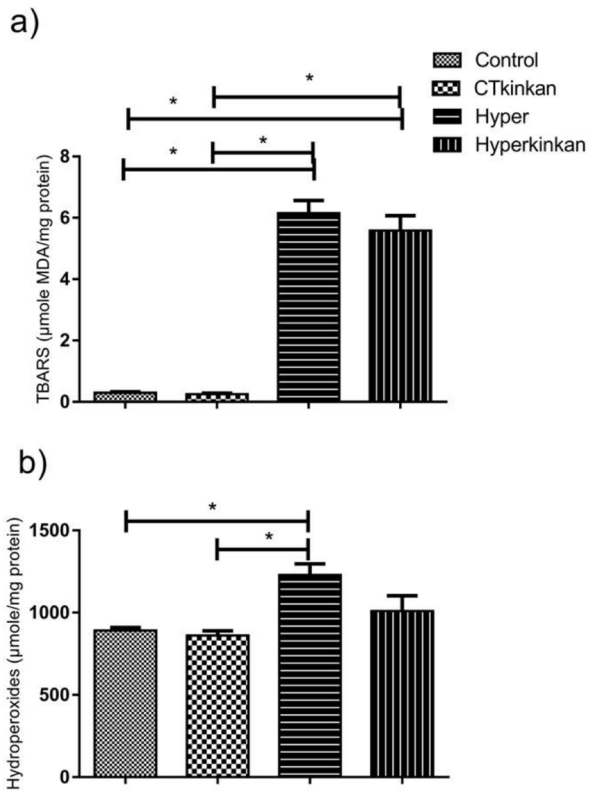


**Figure 2. Effect of kinkan orange intake on hepatic (a) TBARS and (b) hydroperoxides levels of rats given Control, CTkinkan, Hyper, and Hyperkinkan diets. The data represents means ± SEM of 10 animals per group. *p < 0.05. *Bars represent statistical significance, p < 0.05, using two-way ANOVA followed by post hoc Bonferroni test.**

our study, rats receiving Hyper diet presented metabolic alterations characterized by increased circulating total cholesterol and triglycerides, reduced HDL-cholesterol levels and ectopic fat deposition in the liver, in agreement to some studies reported before using male or female (Fischer / Wistar) rats (Abreu et al. 2014, Baldissera et al. 2017, Hassan et al. 2011). Administration of kinkan orange along with hypercholesterolemic diet (Hyperkinkan group) modulated serum and hepatic lipid profile and showed its prophylactic potential. These results could be attributed mainly to dietary fiber and phenolic components (Choi 2005, Sadek et al. 2009), that displace cholesterol and decrease the hydrolysis of cholesterol esters, reducing the absorption of dietary and biliary cholesterol in the small intestine (Jesch & Carr 2017). The binding to dietary fiber and phenolic components would make bile acids unavailable in the small intestine as surfactants, thus disturbing lipid emulsification, formation of mixed micelles, and the complete digestion of lipids and their absorption, lowering the levels of circulating triglycerides and the bioavailability of lipophilic nutrients (Capuano 2017).

Mulvihill et al. (2009) observed that western diet decreased hepatic carnitine palmitoyl-transferase 1α (CTP-1α) activity and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) mRNA and reduced fatty acid oxidation in LDL Receptor–Null Mice. The citrus-derived flavonoid, naringin, increased PGC-1α gene expression, which increased mitochondrial DNA, enhanced fatty acid oxidation and corrected liver triglyceride accumulation. Cho et al. (2011) have shown that a 0.003% naringin flavonoid supplementation (equivalent to a glass of grapefruit juice) in male Long-Evans hooded rats fed a control diet increased the expression of CTP-1α and uncoupling protein 2 (UCP2), that was associated with the increase in their regulator peroxisome proliferator-activated receptor alpha (PPARα), leading to lower levels of serum and liver triglycerides.

HDL is an important protective factor against coronary heart disease, and it exerts various potentially antiatherogenic properties, including the mediation of reverse transport of cholesterol (RCT) from cells of the arterial wall to the liver and steroidogenic organs (von Eckardstein et al. 2001). These properties have made HDL metabolism an interesting target for pharmacological intervention in atherosclerosis (Nofer et al. 2002). Cholesterol could be transferred from HDL to LDL by

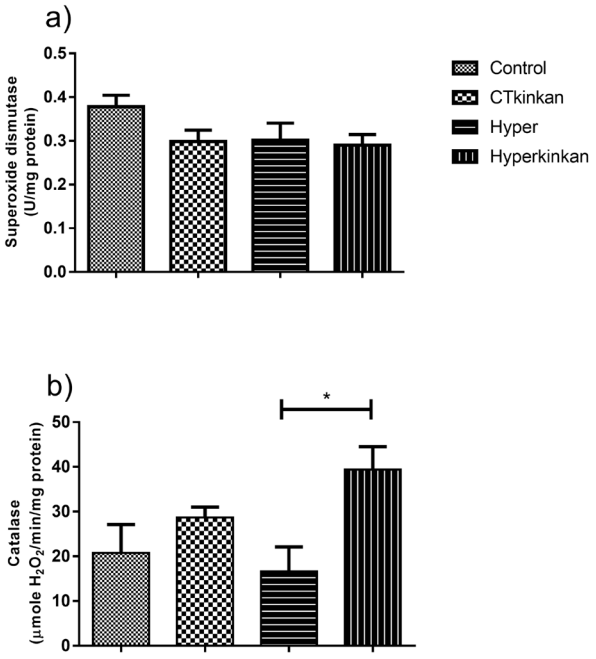


**Figure 3. Effect of kinkan orange intake on hepatic (a) superoxide dismutase (SOD) and (b) catalase activities of rats given Control, CTkinkan, Hyper, and Hyperkinkan diets. The data represents means ± SEM of 10 animals per group. *p < 0.05. *Bars represent statistical significance, p < 0.05, using two-way ANOVA followed by post hoc Bonferroni test.**

cholesterol ester transfer protein (CETP), which is a plasma glycoprotein that facilitates the exchange of cholesteryl esters and triglycerides between HDL and apolipoprotein B-containing lipoproteins (VLDL and LDL). CETP then plays an important role in reverse cholesterol transport (Malhotra et al. 2020). However, mice and rats are CETP-deficient species and transport plasma cholesterol mainly in HDL, unlike humans, where LDL is the main plasma cholesterol transporter (Mindham & Mayes 1991, Liang et al. 2020).

Our results confirmed those found by Lemieux et al. (2005) in female Sprague-Dawley rats fed a cholesterol-free diet, which is that HDL is the major cholesterol carrier, and our study also showed that animals receiving kinkan orange with hypercholesterolemic diet (Hyperkinkan group) had their HDL-C levels increased.

In addition to transporting cholesterol and lipids in the RCT pathway, HDL transports a diverse group of proteins, small RNAs, bioactive lipids, and many other small molecules, that may confer many of alternative HDL functions, including anti-thrombotic, anti-apoptotic, anti-inflammatory, anti-oxidative, anti-infectious, and pro-vasodilatory capacities (Linton et al. 2019). In this way, HDL-C levels alone do not represent HDL particle numbers or HDL function (e.g. cholesterol efflux capacity). Although not having studied the particularities of the circulating HDL in the present study, we believe that animals supplemented with kinkan orange could have HDL particles enriched with bioactive compounds, potentiating the beneficial functions of HDL in hypercholesterolemic conditions. The present study did not focus on the structure and components of HDL, which could be useful to understand the real effects of kinkan orange on the increase in HDL-C for the prevention and treatment of cardiovascular diseases (Ahn & Kim 2016).

After feeding Sprague-Dawley male rats with a high-cholesterol diet (1%) for two weeks, Lasser et al. (1973) found that a new lipoprotein fraction containing low density and high density lipoproteins appeared between the density range of 1.006 and 1.030. The lipoprotein found in the HDL range (between 1.070 and 1.21) was also decreased, but the total HDL protein did not change, suggesting that HDL shifted in part to a lower density class. The authors found that in hypercholesterolemic condition, the increase in lower density lipoproteins was accompanied by a decrease in HDL. Besides that, low density lipoprotein (LDL) consists of several subclasses of particles with different sizes and densities, including large buoyant, intermediate and small dense (sd) LDLs. sdLDL has a greater atherogenic potential than that of other LDL subfractions (Ivanova et al. 2017). In this way, we believe that

these changes in lipoprotein density profile could explain the new way of transporting the excess of cholesterol in hypercholesterolemic group.

Oxidative stress plays an important role in disease pathophysiology of hypercholesterolemia due to increased hepatic production of ROS (Abreu et al. 2014, Baldissera et al. 2017, Ben Gara et al. 2017, Hassan et al. 2011). In this present study, hypercholesterolemia was able to increase lipid peroxidation and probably ROS production, demonstrated by the increase of hepatic TBARS and hydroperoxides levels, respectively, without changing SOD and catalase activities, different from the results found by other authors (Baldissera et al. 2017, Ben Gara et al. 2017, Hassan et al. 2011), which related a decrease in SOD, catalase and glutathione peroxidase activities. The study of Baldissera et al. (2017) on female Wistar rats concluded that the increase on lipid peroxidation possesses pro-inflammatory and cytotoxic properties, that contribute to the initiation and progression of atherosclerosis. Interestingly, we found that the administration of kinkan orange stimulates the hepatic anti-oxidant capacity evidenced by the increase of catalase activity and the normalization of hydroperoxides contents in hypercholesterolemic rats, which possibly could be attributed to some natural antioxidant components, such as phenolic compounds and vitamin C. As shown by other authors, phytochemicals are various biological active compounds which have antioxidant and anti-inflammatory activities based on biochemical interactions with target enzymes or proteins (Sierra-Campos et al. 2020, Rana et al. 2019). They bind antioxidant enzymes (catalase, dismutase superoxide and glutathione peroxidase) with different binding affinities and they are responsible for the effects on the antioxidant activity (Rana et al. 2019). Catalase is known for its ability to bind hydrogen peroxide with high affinity, degrade it into water and oxygen, being the main regulator of its cellular concentration (Sierra-Campos et al. 2020). In this way, we suppose that some phytochemicals found in kinkan orange may have a higher catalase binding affinity, acting as agonists and increasing the activity of this enzyme, mainly in hypercholesterolemic condition.

The present study is the first that has showed the effect of kinkan orange on liver and kidney functions in hypercholesterolemic rats. We found that hyperlipidemia was accompanied by the increase in the amount of liver toxicity in blood, due to the elevation of serum ALT, as reported before (Abreu et al. 2014, Ben Gara et al. 2017, Hassan et al. 2011). Renal function, assessed by the levels of serum creatinine, was also impaired in male Wistar rats (Faran et al. 2019). Kinkan orange consumption did not cause liver or kidney toxicity, reflected by the reduced serum AST, ALT, urea and creatinine levels in hypercholesterolemic condition, suggesting a potential protective effect in liver function. These findings highlighted possibly the efficacy of kinkan orange phenolic compounds as hepatic-protectant in hypercholesterolemic toxicity mainly through suppressing the oxidative status as well as improving metabolic profile.

## CONCLUSIONS

Dietary supplementation with 5% of kinkan orange improved lipid profile and had a beneficial effect against oxidative damage in rats submitted to hypercholesterolemic diet. Our findings raise the possibility that kinkan orange can be used as adjuvant in lipid abnormalities and it is a potential food for cardiovascular protection.

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All authors have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data.