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RESEARCH ARTICLE

Mangifera indica L. extract (Vimang®) reduces plasma and liver cholesterol and leucocyte oxidative stress in hypercholesterolemic LDL receptor deficient mice

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Abstract

Cardiovascular diseases are major causes of death worldwide. Beyond the classical cholesterol risk factor, other conditions such as oxidative stress are well documented to promote atherosclerosis. The *Mangifera indica* L. extract (Vimang®) was reported to present antioxidant and hypocholesterolemic properties. Thus, here we evaluate the effects of Vimang treatment on risk factors of the atherosclerosis prone model of familial hypercholesterolemia, the LDL receptor knockout mice. Mice were treated with Vimang during 2 weeks and were fed a cholesterol-enriched diet during the second week. The Vimang treated mice presented significantly reduced levels of plasma (15%) and liver (20%) cholesterol, increased plasma total antioxidant capacity (10%) and decreased reactive oxygen species (ROS) production by spleen mononuclear cells (50%), $P < 0.05$ for all. In spite of these benefits, the average size of aortic atherosclerotic lesions stablished in this short experimental period did not change significantly in Vimang treated mice. Therefore, in this study we demonstrated that Vimang has protective effects on systemic and tissue-specific risk factors, but it is not sufficient to promote a reduction in the initial steps of atherosclerosis development. In addition, we disclosed a new antioxidant target of Vimang, the spleen mononuclear cells that might be relevant for more advanced stages of atherosclerosis.

Keywords: atherosclerosis; hypercholesterolemia; leucocytes; Mangifera indica L; oxidative stress

Introduction

Cardiovascular diseases remain as a major cause of death in the world (GBD-2015, 2016). The atherosclerosis is the main mechanism underlying ischemic heart diseases and stroke as well as other peripheral vascular diseases. Genetic factors and lifestyle have been extensively associated with atherosclerosis. Nevertheless, elevated plasma cholesterol levels, particularly LDL-cholesterol, have been the unique variable that is sufficient to induce atherosclerosis independently of other risk factors in humans and animal models (Glass and Witztum, 2001). LDL-cholesterol levels and oxidative stress are causally related with the genesis of atherosclerosis (Madamanchi et al., 2005; Steinberg, 2009). The cellular sources of reactive oxygen species (ROS) are multiple, including NAD(P)H oxidase, xanthine oxidase, lipoxygenase, and cyclooxygenase systems, mitochondrial electron transport chain and autoxidation of diverse molecules. We have recently shown the relevance of mitochondrial derived reactive oxygen species (mitoROS) for atherosclerosis. We showed in hypercholesterolemic mice that mitoROS correlates with the severity of atherosclerosis in young mice and during aging (Dorighello et al., 2016, 2017).

Several studies employing antioxidant treatments in humans (Kritchevsky et al., 1995; Stephens et al., 1996) and animal models (Koga et al., 2004; Kurosawa et al., 2005) were successful in reducing atherosclerosis. However, other

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Abbreviations: ALP, Alkaline Phosphatase; ALT, Alanine transaminase; AST, Aspartate transaminase; DCF, Dichlorofluorescein; GSH, Reduced glutathione; H_2DCF-DA, ′,7′-dichloro-dihydro-fluorescein diacetate; LDLr−/−, LDL receptor deficient mice; ROS, Reactive oxygen species; TBARS, Thiobarbituric acid reactive substances

studies have shown the opposite effect, antioxidant treatments that actually increased atherosclerosis in humans and animals (Zhang et al., 1997; Bird et al., 1998). One important point in these contradictory results that must be taken into account is the type of the antioxidant used. Most studies use antioxidants against 1e-oxidants (radicals), which offer little protection against 2e-oxidants (Stocker and Keaney, 2004). *Mangifera indica* L. extract (Vimang®), composed of a mix of polyphenols, terpenoids, steroids, fatty acids and microelements, present antioxidant action in vitro against 1e-oxidants (hydroxyl radical) and 2e-oxidants (hypochlorous acid) (Núñez-Sellés et al., 2007). In humans, it was reported that Vimang daily ingestion by elderly promoted reductions in serum oxidative stress markers (Pardo-Andreu et al., 2006). In addition, 1 week treatment of hypercholesterolemic LDL receptor knockout (LDLr−/−) mice with Vimang resulted in reduced liver mitochondria H_2O_2 (2e-oxidants) formation and susceptibility to mitochondria permeability transition (Pardo-Andreu et al., 2008b), a condition that may lead to cell death. Besides the antioxidant effect, *Mangifera indica L.* extracts have a hypocholesterolemic property as shown in treated normoglicemic and diabetic rats (Anila and Vijayalakshmi, 2002; Muruganandan et al., 2005; Gururaja et al., 2017).

We have previously demonstrated that mitochondria from various tissues of the LDLr−/− mice, a model of familial hypercholesterolemia, presented increased generation of global ROS and H_2O_2 (2e-oxidants) with normal levels of superoxide anion radical (1e-oxidants) (Oliveira et al., 2005; Paim et al., 2008). Thus, considering the antioxidant and hypocholesterolemic properties of Vimang, in this study we evaluate its effects on systemic and tissue risk factors as well as the very early phase of atherosclerosis development.

Material and methods

Animals and protocols

LDL receptor deficient mice (LDLr−/−) (B6.129S7-Ldlr <tm1Her./J, homozygous for Ldlr <tm1Her, stock number 002207) were purchased from The Jackson Laboratory (Bar Harbor, ME) in 2009 and have been maintained in strictly SPF conditions in the Multidisciplinary Center for Biological Investigation in Animal Science (CEMIB) of the State University of Campinas (Unicamp). When the mice reach the age of 4 weeks they were transferred to the Conventional Animal Facility in the Department of Structural and Functional Biology at Unicamp. Mice are then maintained in a temperature controlled room ($22 \pm 1°C$), with 12 h light/dark cycle, with 15 cycles of air changes per hour, with free access to food (standard laboratory rodent chow diet, Nuvital CR1, Colombo, PR, Brazil) and filtered water. They are then enrolled in the experimental protocol, which was approved by the Ethical Committee for the Use of Animal (CEUA/Unicamp, protocol #1101-2) and by the Internal Biosecurity Committee (CIBio-IB/Unicamp, protocol # 2008/02). Four-month-old male LDLr−/− mice were treated with Vimang during 2 weeks and fed with a cholesterol-enriched diet (AIN-93G, 7 g% fat, and 0.21 g% cholesterol) during the second week. LDLr−/− mice were randomized into experimental (Vimang) and control groups. The Vimang was added in the AIN-93G diet in a concentration (2 g/kg) to reach a daily oral dose of 250 mg/kg of body weight. This dose was previously stablished in a study that showed that one week of 250 mg/kg Vimang was effective to reduce mitochondrial oxidative stress (Pardo-Andreu et al., 2008b). Effective doses for humans are much lower, such as 900 mg/day (Pardo-Andreu et al., 2006). Preparation of *Mangifera indica* L. extract (Vimang®) was previously described (Pardo-Andreu et al., 2008a). The chemical composition of the extract has been reported elsewhere (Núñez Sellés et al., 2002) and contained phenolic acids (gallic acid, 3,4-dihydroxy benzoic acid, benzoic acid), phenolic esters (gallic acid methyl ester, gallic acid propyl ester, benzoic acid propyl ester), flavan-3-ols (catechin and epicatechin) and the xanthone mangiferin, which is the major component of Vimang® (16%).

Plasma biochemical analysis

Blood samples were obtained from the retro-orbital plexus of anesthetized and overnight fasted mice. Total cholesterol and triglycerides were measured in fresh plasma using standard commercial kits (Roche-Hitachi®, Germany). Glucose levels were measured using a hand-held glucometer (Accu-Chek Advantage, Roche Diagnostic®, Switzerland). The total antioxidant capacity was measured following the manufacturer's instructions (Cayman Chemical, USA). The assay determined the capacity of the antioxidants in the sample to prevent the substrate (ABTS) oxidation compared with the capacity of a known antioxidant (Trolox, a water-soluble tocopherol analogue). The plasma urea, creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transaminase (AST) were determined using an automated modular analyzer PP (Hitachi) with Roche reagents (Roche Diagnostic®, Germany). $CuSO_4$-induced plasma thiobarbituric acid reactive substances (TBARS) concentrations were determined after exposing plasma to 0.1 mM $CuSO_4$ at 37°C for 5 h. Then, 100 μL of oxidized plasma was incubated with 200 μL of 0.7% thiobarbituric acid in 0.05 M NaOH and 60 μL of 50% trichloroacetic acid. Samples were incubated in boiling water for 30 min, followed by centrifugation at 664*g* for 15 min. The standard curve was prepared using serial dilutions of 0.05 mM 1,1,3,3-tetramethoxypropane. The optical densities

of samples and standard curve were measured in a microplate reader at 532 nm.

Body composition and tissue lipids analyses

The epididymal adipose tissue, liver, and spleen fresh masses were determined gravimetrically. Liver lipids were extracted with chloroform/methanol (2:1) solution and dried with nitrogen gas. The liver content of cholesterol and triglycerides were determined using colorimetric-enzymatic assays (Roche-Hitachi®, Germany) after dissolving the lipid extracts in a triton-containing buffer (0.1 M potassium phosphate, pH 7.4, 0.05 M NaCl, 5 mM cholic acid, 0.1% Triton® X-100).

Isolation of mouse liver mitochondria

Mitochondria were isolated by conventional differential centrifugation at 4°C as previously described (Kaplan and Pedersen, 1983). The livers were homogenized in 250 mM sucrose, 1 mM EGTA, and 10 mM Hepes buffer (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was suspended in 250 mM sucrose to a final protein concentration of 80–100 mg/mL. The protein concentration was determined by a modified Biuret assay. The procedure was done by a co-author unaware of the sample identification (blind).

Isolation of spleen mononuclear cells

Fresh spleens were softly homogenized in a Douncer manual homogenizer. The homogenates were overlaid onto a Ficoll-Paque TM PLUS layer with the density adjusted to 1.076 g/mL and centrifuged at 1000*g* at room temperature for 25 min. The interface cell layer was recovered, washed twice in PBS and centrifuged at 500*g* for 10 min (Böyum, 1967). The procedure was done by a co-author unaware of the sample identification (blind).

Reactive oxygen species production (ROS)

ROS production was monitored using the membrane-permeable fluorescent dye 2′,7′-dichloro-dihydro-fluorescein diacetate (H_2DCF-DA). Briefly, 1 μM H_2DCF-DA was added in a 1 mL cuvette with constant stirring at 37°C containing the mitochondria suspension (0.5 mg/mL) or spleen mononuclear cells (10^6 cells/mL). The fluorescence signal was recorded at the excitation/emission wavelength pair of 488/525 nm using a fluorimeter (Hitachi, model F4500). Calibration was performed with known concentrations of dichlorofluorescein (DCF), which is the product of H_2-DCF oxidation. Data were obtained by a co-author unaware of the sample identification (blind).

NADPH oxidation rates

The redox state of pyridine nucleotides was measured as previously described (Oliveira et al., 2005) in the mitochondrial suspension (1 mg/mL) by following the fluorescence signal in a Hitachi F-4010 spectrofluorometer using excitation and emission wavelengths of 366 and 450 nm, respectively, and a slit width of 5 nm. The extent of pyridine nucleotide oxidation was calculated as a function of the fluorescence increase induced by isocitrate addition. Internal calibration was done by the addition of known amounts of NADH. Data were obtained by a co-author unaware of the sample identification (blind).

Histological analysis of atherosclerosis

In situ perfused hearts were excised and embedded in Tissue-Tek® OCT compound (Sakura, USA), frozen at −80°C, cut in 10 μm-sections along 480 μm aorta length from the aortic valve leaflets and stained by Oil red O and counterstained with light green (Paigen et al., 1987). The lipid-stained lesions were quantified as previously described (Rubin et al., 1991) using the *Image J* (1.45 h) software. The slides were prepared and images were quantified without group identification (blindly).

Statistical analyses

The results are presented as the means ± SE. Two mean comparisons were analyzed by Student t test. For correlation analyses, the Spearman's univariate correlation test and Partial (adjusted) correlation were performed using the softwares GraphPad Prism 5.0 and SPSS Statistics 14.0. The level of significance was set at $P < 0.05$.

Results

In this study, we evaluated the effects of the Vimang treatment on tissue and mitochondria redox state, plasma and liver lipids and early phase of atherosclerosis development in the LDL receptor deficient mice (LDLr−/−). Mice were fed with a low fat diet enriched with cholesterol to induce the experimental atherosclerosis.

Vimang treatment did not modify the body weight and the relative weight of adipose tissue, liver, and spleen of the LDLr−/− mice (Table 1). Plasma glucose and renal (urea and creatinine) and hepatic (AST, ALT, and alkaline phosphatase enzymes) function markers also were not changed by Vimang treatment, indicating no renal or liver toxic effects (Table 1). On the other hand, the total antioxidant capacity of plasma was 10% ($P < 0.05$) increased in the LDLr−/− mice treated with Vimang, while the $CuSO_4$-induced TBARS plasma levels, a lipid peroxidation

Table 1 Body and tissue masses, plasma glucose, TBARS and antioxidant levels, renal and hepatic function markers in LDLr−/− mice treated or not with Vimang.

Parameters	Control	Vimang
Body weight (g)	23.2 ± 0.7	24.4 ± 0.6
Epididymal adipose tissue (% of body weight)	2.36 ± 0.30	2.58 ± 0.19
Liver (% of body weight)	4.39 ± 0.13	4.48 ± 0.11
Spleen (% of body weight)	0.21 ± 0.01	0.22 ± 0.01
Glucose (mg/dL)	76.5 ± 4.1	80.7 ± 2.7
TBARS (μmol/L)	492 ± 27	491.4 ± 16
Total antioxidants (mM)	0.41 ± 0.01	0.45 ± 0.01*
ALT (U/L)	48.8 ± 5.3	54.4 ± 9.4
AST (U/L)	127.5 ± 11.2	129 ± 10.7
ALP (U/L)	88.2 ± 6.5	81.6 ± 2.6
Urea (mg/dL)	71.4 ± 1.9	72.2 ± 2.9
Creatinine (mg/dL)	0.09 ± 0.01	0.10 ± 0.01

Data are mean ± SE (n = 9–10/group). Vimang treatment: 250 mg/kg of BW per day during 2 weeks (ALT, Alanine transaminase; AST, Aspartate transaminase; ALP, Alkaline Phosphatase). *$P < 0.05$, Student t test (Vimang vs. control).

marker, were similar in treated and untreated groups (Table 1).

The Vimang treated group presented a reduction of 15% ($P < 0.05$) in the plasma cholesterol levels and of 20% ($P < 0.05$) in the liver cholesterol content. No differences were observed in the triglyceride levels (Figure 1).

The antioxidant properties of Vimang have been shown in previous studies (Sánchez et al., 2000; Pardo-Andreu et al., 2006), therefore, we investigate the potential of Vimang in modulating cell and organelle redox state. We studied isolated liver mitochondria and whole linfomononuclear cells obtained from spleen. The first is representative of most body tissues and the second is a cell population particularly relevant for atherosclerosis. In the isolated liver mitochondria, the reactive oxygen species (ROS) production and the NADPH content, the major mitochondrial reducing equivalents source, were not influenced by Vimang treatment (Table 2). However, in spleen mononuclear cells, ROS production was markedly reduced by Vimang treatment (50%, $P < 0.05$).

Despite the benefits of Vimang regarding reduction of plasma and liver cholesterol and lymphocyte ROS levels, the average size of early atherosclerotic lesions in the aorta root of treated mice was similar to that observed in non-treated LDLr−/− mice (Figure 2).

Discussion

Mangifera indica L. extract biological activities include effects as antioxidant, antidiabetic, anti-inflammatory, and immunomodulatory (Nunez Selles and Villa, 2015). However, no studies evaluated directly the effects of *Mangifera indica* L extracts on the atherosclerosis development. This study was designed to investigate the Vimang effects on the early steps of atherosclerosis development. Thus, the effects of Vimang were verified after a short (2 weeks) experimental period, including only 1 week of a cholesterol enriched diet. Although there is no reliable animal model to study regression of atherosclerotic lesions (Kapourchali et al., 2014), the apolipoprotein (apo) E-knockout and the LDL receptor knockout (LDLr−/−) mice have been extensively used and contributed to the advance the knowledge about the progression of atherosclerotic lesions (Kapourchali et al., 2014). We have shown that the LDLr−/− model develop spontaneous lesions even in the absence of an atherogenic diet (Dorighello et al 2017). Therefore, the animal model and the protocol are appropriated to see the potential preventive effects in the early steps of atherogenesis if the compound is effective.

The liver content and plasma levels of cholesterol were significantly reduced by Vimang treatment. This is in accordance with previous studies that showed the hypocholesterolemic effects of mangiferin, Vimang's main component in diabetic rats (Muruganandan et al., 2005) and rats fed with a cholesterol-enriched diet and treated with *Mangifera indica* L. flavonoids (Anila and Vijayalakshmi, 2002; Gururaja et al., 2017). The reduction of cholesterol in the serum and tissues of treated rats was shown to be a consequence of enhanced fecal excretion of acid and neutral steroids (Anila and Vijayalakshmi, 2002). This seems to be exactly the case in the present study, since we observed decreased serum and liver cholesterol in the mice under cholesterol feeding. Our results suggest that Vimang may have increased dietary cholesterol excretion via liver.

LDLr−/− mice treated with Vimang had an increase in the total antioxidant capacity of their plasmas and a marked reduction of ROS production by their spleen lymphocytes.

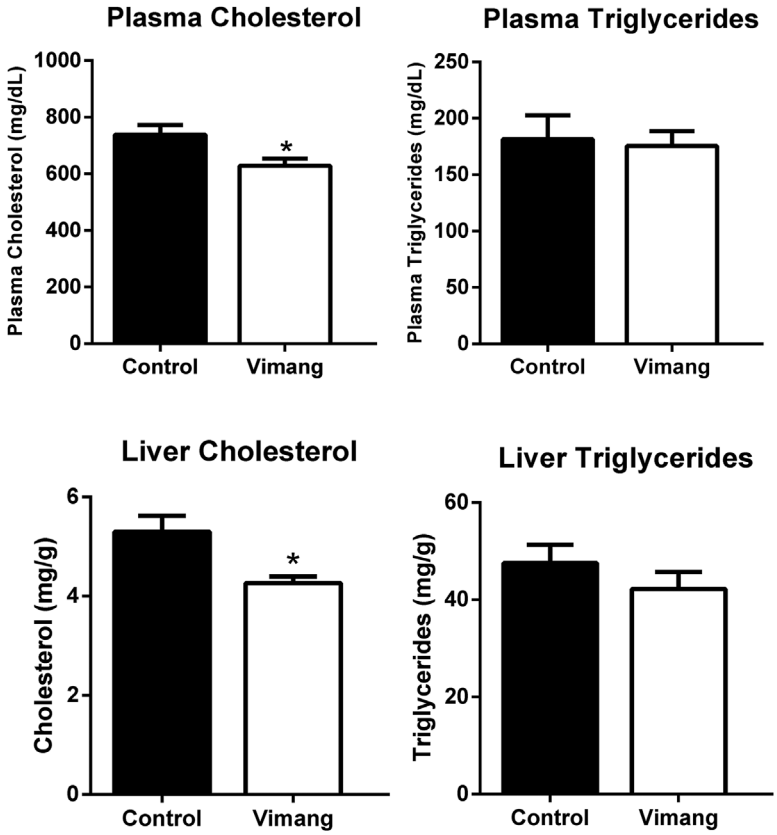

Figure 1 Plasma and liver cholesterol and triglyceride levels of LDLr−/− mice treated with Vimang during 2 weeks and fed with a cholesterol-enriched diet (0.21% g) during the second week. The Vimang was added to diet (2 g/kg) to reach the daily dose of 250 mg/kg of body weight per day. N = 9–10/group. $^{*}P < 0.05$ related to control, Student t test.

Accordingly, other previous studies have also shown antioxidant properties of Vimang or some of its fractions in in vitro and in vivo systems. For instance, Vimang promoted reduction in ROS production by peritoneal macrophages (Sánchez et al., 2000), inhibition of Fe^{2+}-citrate-induced lipoperoxidation in isolated rat liver mitochondria (Pardo-Andreu et al., 2005), reduction of H_2O_2 generation by liver mitochondria of LDLr−/− mice (Pardo-Andreu et al., 2008b) and reduction of lipid and protein oxidized products in serum and liver of treated rats (Roche and Pérez, 2012).

In theory, mononuclear cells that produce less ROS could be less responsive to macrophage differentiation and foam cell formation and would cause less LDL oxidation, particularly in the sub-endothelial milieu. Although the two-week treatment with Vimang reduced the plasma and liver cholesterol and the mononuclear cell ROS production in the LDLr−/− mice, the atherosclerosis lesion size stablished in their aorta root (predilection site) was not reduced. Several reasons may account for these negative results. Since these initial lesions are too small and the disease progresses exponentially, it is possible that the period of the treatment was too short to evidence a larger effect of Vimang. Another point is that lesions were measured only by lipid staining. Perhaps earlier features of initial lesions, such as monocytes infiltration, would have been more sensitive to detect changes in such a short time protocol.

Table 2 Tissue redox state parameters measured in isolated liver mitochondria and spleen mononuclear cells of LDLr−/− mice treated or not with Vimang.

Parameters	Control	Vimang
Liver mitochondria ROS production (ηM DCF/mg protein.min-1)	2.65 ± 0.3	2.70 ± 0.4
Liver mitochondria NADPH oxidation (ηM NADPH/mg protein.min$^{-1)}$	1.66 ± 0.1	1.75 ± 0.1
Spleen mononuclear cells ROS production (ηmol DCF/10^{6}cells.min^{-1})	135 ± 28	69 ± 2.5*

Data are mean ± SE (n = 9–10/group). Vimang treatment: 250 mg/kg of BW per day during 2 weeks. $^{*}P < 0.05$, Student t test (Vimang vs. control).

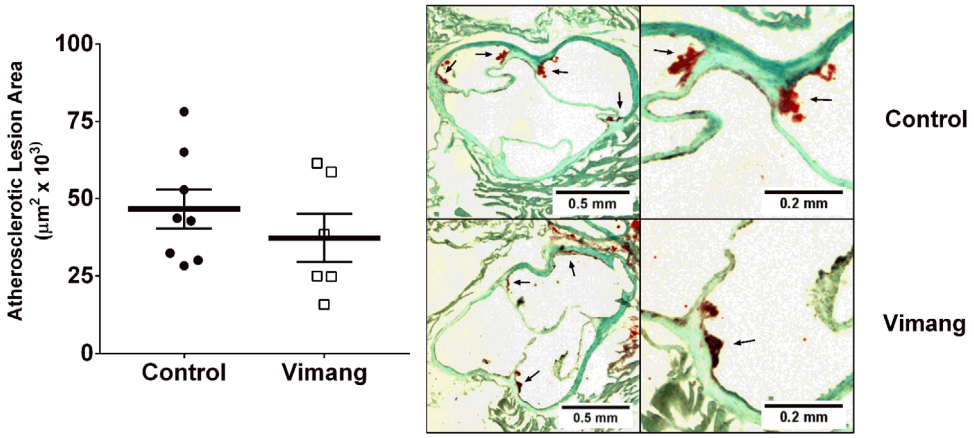

Figure 2 Atherosclerosis lesion areas and representative images of lipid stained aorta root lesions of LDLr−/− mice treated with Vimang during 2 weeks and fed with a cholesterol-enriched diet (0.21% g) during the second week. The aortic roots were cryo-sectioned and stained for lipids with oil red-O (arrows) and counterstained with light green. N = 8–9/group.

However, we think that hydrophilic property of Vimang might be the most important limiting factor. Strong evidence supports the critical role of oxidized low-density lipoprotein in initiation and progression of atherosclerosis (Steinberg, 2009).The low lipophilicity of Vimang components (e.g., Vimang is prepared from aqueous extracts of *Manigifera indica* L stem bark) could limit their access to LDL, hampering the protection of the lipoproteins from oxidation. Indeed, the natural extract did not reduce TBARS (lipid peroxidation), although it increased total plasma antioxidant capacity. This latter effect could reflect the higher contribution of hydrophilic antioxidants after Vimang supplementation that counteract some but not all the oxidative events involved in the development of atherosclerotic lesions. Despite Vimang treatment was not sufficient to promote a reduction in the initial steps of atherosclerosis development, its use as a complementary therapy to prevent atherosclerosis in patients with hypercholesterolemia may be suggested, particularly for those poor responders to hypolipidemic drugs.

In conclusion, the *Mangifera indica* L. extract Vimang treatment of LDLr−/− mice promoted protective effects on leucocyte ROS production and plasma and tissue concentrations of cholesterol. However, it was not sufficient to promote a reduction in the initial steps of atherosclerosis development, although it does not exclude a possible role in more advanced stages of the disease. The novelty of these findings is the identification of a new antioxidant target of Vimang, the spleen mononuclear cells that might be relevant for the long-term development of atherosclerosis.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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