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Moringa oleifera leaves extract regulate the activity of nitric oxide synthases and paraoxonase 1 in diabetic rat

Abstract

This study was aimed at evaluating the effects of *M. oleifera* leaves extract on biochemical physiological parameters, nitric oxide synthase and paraoxonase 1 activities of diabetic model rats. Eighteen Wistar rats were equally split into three groups as follow: control (water), alloxan administration (120 mg/kg b.w), and *M. oleifera* extract treatment (200 mg/kg b.w., daily for twenty-one days), with six rats per group. Glucose, triglycerides, glycated hemoglobin, advanced end products, nitrites were measured in serum. In parallel, nitric oxide synthase (NOS) and paraoxonase 1 (PON1) activities were tested in heart. The results showed that alloxan induces alterations in nitric oxide homeostasis and inhibited the activities of constitutive NOS and PON1, resulting in oxidative stress. While the levels of AGEs and HbA_{1c} were elevated in the serum of alloxan-diabetic animals. Oral administration of *M. oleifera* in diabetic animals reduced the glucose, triglycerides, AGEs and HbA1c. In addition, *M. oleifera* treatment significantly rescued the cNOS and PON1 compared to nontreated diabetic group. Surprisingly, we observed a significantly activation of PON1 activity in the *M. oleifera* treatment compared to control group. However, are necessary to further elucidate the activation mechanism. These findings provide a perspective on the cardioprotective effects of diabetic conditions.

Keywords: diabetes mellitus, paraoxonase 1, nitric oxide synthase, *moringa oleifera*, heart

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Erick Sierra-Campos,[1] Mónica A Valdez-Solana,[1] Jesús R Pérez-Velázquez,[1] Guadalupe García-Arenas,[2] Alfredo Téllez-Valencia,[3] Claudia Avitia-Domínguez[3]

[1]Faculty of Chemical Sciences, Juarez University of the State of Durango Campus Gómez Palacio, Mexico
[2]Faculty of Health Sciences, Juarez University of the State of Durango Campus, Mexico
[3]Faculty of Medicine and Nutrition, Juarez University of the State of Durango Campus Durango, Mexico

Correspondence: Erick Sierra Campos, University Juarez of Durango State, Av. Articulo 123 S/n Fracc. Filadelfia. CP. 35000, Gomez Palacio, Durango, Mexico, Tel +5287 1715 8810, Email ericksier@gmail.com

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Abbreviators: NO•, nitric oxide; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; $O_2^{\bullet-}$, superoxide anion; Hb, hemoglobin; HbA_{1c}, glycated hemoglobin; HbO_2, oxyhemoglobin; AGEs, advanced glycation products; PON 1, paraoxonase 1; HDL, high density lipoproteins; LDL, low density lipoproteins; NOX2, NADPH oxidase 2; ROS, reactive oxygen species; L-arg, L-arginine; cNOS, constitutive nitric oxide synthase; BH_4, tetrahydrobiopterin; $BH_3^{\bullet}$, protonated trihydrobiopterin radical cation; BH_2, dihydrobiopterin; ONOO•, peroxynitrite; CR, red cells

Introduction

Diabetes mellitus is the major health problem in Mexico. Close to 20% of the preventable deaths in our country are caused by diabetes and related metabolic diseases.[1,2] Diabetes mellitus is associated with accelerated atherosclerosis development and with increased risk for cardiovascular disease. Hyperglycemia accelerates atherosclerosis by several mechanisms, including the induction of vascular dysfunction, increasing oxidative stress, increasing lipid peroxidation of lipoproteins and of arterial cells, including macrophages, which may then lead to enhanced macrophage foam cell formation, the hallmark of early atherosclerosis development.[3,4]

It has been well recognized that nitric oxide (NO) is an imperative regulator of the cardiovascular system and is a critical mechanism in preventing the pathogenesis and progression of the cardiovascular complications.[5,6] In addition, several studies suggest that NO production is reduced in diabetes and that the decrease of NO may be related to the pathogenesis of diabetic endothelial damage.

NO is synthesized by the members of the NO synthase (NOS) family by the oxidation of L-arginine and NADPH by oxygen to yield L-citruline and NO.[7] Moreover, NO synthase activity is known to be constitutively low in the normal heart. However, it may dramatically increase when the inducible NO synthase (iNOS) is expressed as in inflammatory reactions or septic shock.[8] However, it has been reported that diabetes causes a higher expression of iNOS in cardiovascular tissues of diabetic rats.[9] Therefore, NO is highly reactive and acts as signaling molecule at low concentration and generates nitrosative stress at high concentration. Thus, both lack and excess of NO production in diabetes can have various important implications on heart function in the body.

The paraoxonases family (PONS, including PON1, PON2 and PON3) are calcium dependent esterases with antioxidant properties,[10] with enzymatic activities targeted towards a broad range of substrates. Although PONs physiological substrates were not identified yet, several studies showed that PONs are lipo-lactonases, which hydrolyze specific oxidized phospholipids, including products of enzymatic and nonenzymatic oxidation of arachidonic and docosahexaenoic acid, as well as, N-acyl-homoserine lactones and lipo-lactones. Earlies results showing the transfer of PON1 between membranes[11] and the expression of PON1 in multiple mouse tissues[10] suggested that PON1 is transferred via HDLs from the liver to tissues where its activity is needed. Hence, inhibition or reduction of PON results in CVD and endothelial cell apoptosis.[12] Thus, one of the treatment options for preventing CVD and vascular damage due to apoptosis is to elevate PON1 and NOS activities.

Moringa oleifera has been shown to induce cardioprotective mechanism in coronary artery rings. Some et al.[13] determined the effect of ethanolic and aqueous extract on vascular relaxation in porcine coronary artery rings and showed that *Moringa oleifera* induce an endothelium dependent and endothelium independent vasorelaxation effect. In addition, several lines of evidence suggest that Moringa fruit inhibits LPS-induced NO/iNOS expression through suppressing the NF-κB activation in RAW264.7 cells[14,15] and other inflammatory markers such as prostaglandin E_2 and interleukins.[16] However, little is known about the potential role of *Moringa oleifera* in activation of

NO synthase. These effects were at least partly mediated through of the eNOS-NO-cGMP pathway. In addition, a recurring explanation for the therapeutic actions of *M. oleifera* medication is the relatively high antioxidant, hypolipidemic and anti-atherosclerotic activities of its leaves,[17] no data on the effect of this medicinal plant on nitric oxide homeostasis are available. The present study therefore aimed at evaluating the effect of *M. oleifera* leaves extract on the NOS and PON1 activities driving its therapeutic properties in diabetic heart.

Materials and methods

Preparation of the extract

The extract was prepared using 23 g of dry-ground sample and 260mL of 80% methanolic aqueous solution by successive maceration. The mixture was shaken in a magnetic grid at room temperature for 24h and then filtered through Whatman filter paper number 1. The final extract was concentrated on a rotary evaporator, placed in a deep freezer for 24 h and lyophilized to obtain a powdered extract that was kept at −80 °C.

Ethics statement

All experiments were performed in compliance with the guideline for the welfare of experimental animals by the National Institutes of Health and in accordance with the guidelines of Institutional Animal Care. This study was approved by the Institutional Animal Ethics Committee at the Faculty of Health Science, UJED.

Diabetic model and treatment

Alloxan was dissolved in a citrate buffer (0.1M, pH4.5) and intraperitoneally injected (120 mg/kg) to induce diabetes in rats. Rats injected only with citrate buffer served as control. Type 1 diabetes was confirmed evaluating fasting plasma glucose levels after 5days of induction; the inclusion criteria to establish diabetes were 200 mg/dL of fasting plasma glucose (Accu-Chek, Germany). Rats were divided in control (C group), diabetic (D group), and *M. oleifera*-treated diabetic (M group) groups. M group was daily administered with a 200 mg/kg dose of extract by gavage for 3 weeks, and remaining groups were administered with water as vehicle.

Isolation heart cytosolic fraction

The hearts were homogenized three times at high speed (Polytron PowerGen model 125, Fisher Scientific) at 4°C in a homogenizing solution for 20s. The homogenizing solution contained 20 mM Tris-HCl, 200 mM mannitol, 50 mM sucrose, 1mM EDTA, 1 mM PMSF, 1 protease inhibitor tablet, and 0.1% bovine serum albumin (BSA) (pH 7.4). Cellular and nuclear fractions were removed in the pellet by centrifuging at 3,500 rpm for 10min at 4°C. Mitochondria were obtained by centrifuging the supernatant for 10min at 11,000 rpm. The supernatant is referred to as the cytosolic fraction.

Determination of PON1 lactonase activity

For the determination of PON1 lactonase activity in serum, 2-dihydroxy-coumarin was used as substrate according to Billecke et al.[18] with slight modifications. The assay mixture contained 100 µL of 10 mmol/L substrate solution, 5 µL of serum and 1mmol/L $CaCl_2$ in 50 mmol/L Tris buffer, pH 8.0. Product formation was determined spectrophotometrically after 5 min at 270 nm. Molar extinction coefficient is $876M^{-1}cm^{-1}$ was used to calculate the rate of hydrolysis. PON1 activity was monitored in triplicate and corrected for the non-enzymatic hydrolysis and the results are presented as nmol/min/mg protein.

NO measurement by Griess reaction

NO concentration was determined by quantifying the stable end-product of NO, nitrite. A colorimetric assay based on the Griess reaction was used. Briefly, 80µL of serum of each treatment (C, D and M groups) were sequentially mixed with 80 µL of a 1% solution of sulfanilic acid in 5% phosphoric acid and 80 µL of a 0.1% solution of N-(1-naphthyl) ethylenediamine dihydrochloride. The mixture was kept at room temperature for 30 min. The absorbance was measured at 550 nm. Nitrite concentrations were calculated from a standard curve obtained by diluting the sodium nitrite stock solution.

AGEs fluorescence measurement

The formation of total AGEs was assessed by monitoring the production of these fluorescent products of glycated proteins samples and positive control at excitation and emission wavelengths of 370 and 440 nm respectively on spectrofluorometer. The fluorescence of AGEs was expressed as arbitrary units per protein concentration.

Glycosylated hemoglobin (HbA_{1c}) levels

Commercial enzyme-linked immunosorbent assay kit was used to quantify glycosylated hemoglobin levels. The analysis was performed in accordance with instructions provided by the manufacturer (Biocompare, USA).

Statistical analysis

Data obtained from the different experimental groups were compared by one-way ANOVA followed by LSD test for post hoc analysis, using Sigmaplot 12.3 software. Differences with $P<0.05$ were considered significant. Data are presented as mean ± SEM.

Results

Table 1 shows the general parameters of the different experimental groups (control, C; diabetics, D; and diabetics treated with *Moringa oleifera* extract, M). It is interesting to note that the weight of heart in group D was 50% less than the weight of the other treatments (C and M), which could have negative effects on the functionality of the cardiovascular system. Despite this difference, the body weight of the three treatments only changed slightly, with noticeable different at M group. In addition, the heart weight/body weight (HW/BW) ratio decreased almost two times in D group in comparison with C group, by contrast, M group resulted in an increased HW/BW ratio in comparison to the D group. On the other hand, glucose and triglyceride levels were higher in group D compared in the control group. In this regard, group M presented a significant decrease with respect to group D and its values are closer to those reported in the control group.

Table 1 General features of experimental rats

Parameter	C group	D group	M group
Body weight (g)	167.2±8[i]	172±10[i]	184±6
Heart weight (g)	0.73±0.06*	0.48±0.04	0.7±0.02*
Heart/BW ratio (mg/g)	4.36±0.16	2.79±0.2**	3.8±0.1**

Each value represents mean ± SE. *P< 0.05 level of significance of difference between D group and other groups (C and M). **P< 0.05 level of significance of difference between C group and other groups (D and M). [i]P< 0.05 level of significance of difference between M group and other groups (C and D).

Our results showed that levels of glucose and triglycerides increased by 5.2 and 2.6 times, respectively, in the plasma of D group compared to C group (Table 2). In addition, an expected significant

difference was observed in plasma HbA1c levels between D group compared to C group. Similarly, a significant difference was difference between these two groups was also observed in F-AGEs, as they were significantly increased by 8.5 times in the D group and they remained unaffected in the M group. To demonstrate that dysfunction occurred at the level of the vasculature, we performed the measurement of serum nitrites as an indicator of nitric oxide levels. In the Table 2 also shows that the concentrations of nitrites in group D increased 2 times more than in group C, in contrast, M group presented a decrease approximately 3 times lower with respect to the value obtained in D group. In addition, M group presents the lowest levels of nitrites compared to the other study groups. All these data together indicate that the administration of the diabetic agent modified the metabolism of carbohydrates and lipids; this in turn supports endothelial dysfunction and the appearance of cardiovascular complications. A possible explanation for this behavior could be because *M. oleifera* extract is very rich in antioxidant compounds that could modify the redox state of nitric oxide. Therefore, the next aim of this study was to quantify the activity of nitric oxide synthases (eNOS and iNOS) present in heat cytosol fraction.

Table 2 Biochemical parameters of experimental groups

Parameters	C group	D group	M group
Plasma glucose (mg/dL)	80±5	415±26**	125±13**
Plasma Triglycerides (mg/dL)	74±9	198±12**	145±4**
Plasma HbA_{1c} (%)	6.5 ± 0.7*	10.4 ± 2.1	8.3 ± 3.7*
F-AGEs (AU/g protein)x10^5	0.8 ± 0.2*	6.8 ± 1.2	1.7 ± 0.5*
Serum nitrites levels (ppm)	5.66± 0.8[ı]	10.89 ± 0.3**[ı]	2.74 ± 0.5**

Each value represents mean ± SE. *P< 0.05 level of significance of difference between D group and other groups (C and M). **P<0.05 level of significance of difference between C group and other groups (D and M). [ı]P< 0.05 level of significance of difference between M group and other groups (C and D).

Table 3 Heart nitric oxide synthase activity

Groups	Basal activity (nmol/min/mg protein)
C	1.4 ± 0.3
D	12 ± 4.8
M	0.8 ± 0.6
Activation of eNOS activity by BH_4 +calmodulin	
C	7.2 ± 1.8[ı]
D	10.6 ± 2.1**[ı]
M	4.5 ± 0.7**

Each value represents mean ±SE. **P< 0.05 level of significance of difference between C group and other groups (D and M). [ı]P< 0.05 level of significance of difference between M group and other groups (C and D).

Table 4 Serum paraoxonase 1 activity

	C group	D group	M group
PON1 activity (nmol/min/mg protein)	65.83±8.9[ı]	43.2±3.5**[ı]	107.4±7.9**

Each value represents mean ± SE. **P< 0.05 level of significance of difference between C group and other groups (D and M). [ı]P< 0.05 level of significance of difference between M group and other groups (C and D).

Similarly, to the results of Table 2, the M group presented the lowest activity values, which correlates with the fact that the nitrite levels are derived from the activity of the NOS and not from other intracellular sources (Table 3). It is important to note that D group has the highest NOS activity, which corresponds 10times more than C group. As the different isozymes of NO synthase are characterized by their dependence on calcium/calmodulin and by their sensitivity to BH_4. Basal NO activity was first measured in homogenates prepared from hearts. The activity in the presence of a saturating concentration of BH_4 and calmodulin was 7.2 ± 1.8 nmol/min/mg protein in C group. One possible explanation is that during the development of the pathology the expression and activity of the iNOS significantly increases and by on the other hand, the eNOS tends to be uncoupled, which causes the appearance of an oxidative or nitrosative stress. To assess the previous hypothesis, the cytosolic fractions of each treatment were incubated in the presence of BH_4 and calmodulin to favor the activity of the eNOS. It was observed that the addition of both BH_4 and calmodulin favors that the NOS values increase in C and M groups. In contrast, D group does not modify its NOS activity due to the presence of these substrates, which suggests that this activity is of the iNOS while in the other groups (C and M), its activity derives mainly from a constitutive NOS that depends on BH_4 and CaM (Table 3).

The fact that group C and M had a positive effect when adding BH4 and Calmodulin suggests that this condition may favor oxidative stress in the vasculature. In addition, the high activity of the NOSi observed in group D is consistent with the idea that in this pathology a moderate inflammatory process occurs which favors lipid lipoperoxidation and considering the elevated levels of triglycerides observed in Table 2 propose the measurement of a lipoprotein biomarker of its oxidation in the bloodstream. Therefore, in Table 4, it is observed that the activity of paraxonase 1 (PON1) in D group decreased slightly significantly with respect to C group. To our surprise, M group showed an almost double increase in PON 1 activity with respect to C group. These results suggest that *M. oleifera* not only protects the activity of PON 1 but also activates it. However, the activation mechanism is in the study stage. Each value represents mean ± SE. **P<0.05 level of significance of difference between C group and other groups (D and M). [ı]P< 0.05 level of significance of difference between M group and other groups (C and D).

Discussion

Under high glucose conditions, vascular endothelial cells generate high levels of ROS, inflammatory markers and have impaired nitric oxide biosynthesis. Together these factors result in an imbalance between vasoconstriction and vasodilation, as well as an inability to regulate vascular tone characterizing endothelial dysfunction, the hallmark of diabetes.[19] It has been reported that diabetes causes a higher expression of cardiac nitric oxide synthases.[20] These enzymes are responsible of producing NO in the heart, being essential for heart homeostasis and mechanical activity.[21] The enhancement in NO production could be associated with nitration of cytoskeletal proteins, leading to alterations in cardiac contractility.[22] In diabetic hearts, the NOS activity inhibition by L-NMMA or L-NAME, improves cardiac activity, suggesting that both NO and peroxynitrite ($ONOO^-$) could be associated with the inhibition of cardiac contractility.[23]

Our result tends to support that advanced glycation end-products (AGEs) and their signal-transducing receptor interactions induce the formation of ROS, leading to concomitant oxidative stress and vascular inflammation, thereby playing a central role in the pathogenesis of various vascular complications in diabetes.[24] Several studies strongly suggest that glycation of circulating proteins plays a critical and causative role in the pathogenesis of diabetes.[25] Since

circulating erythrocytes (Red cells) are continuously interacting with glycated albumin during hyperglycemic conditions. During circulation, the interaction of glycated albumin with erythrocytes causes cell aggregation[26] and subsequently induce oxidative stress in vessel wall resulting in the development of diabetic complications.[27] Thus, erythrocytes are important in orchestrating the toxic effects of glycated albumin. High glucose leading to increased ROS-induced oxidative stress through PKC-dependent activation of NADPH oxidase in vascular endothelial cells plays a central role in the onset of diabetic micro- and macrovascular disorders.[28] In addition, inducible NO synthase (iNOS) expression and $ONOO^-$ radical formation are significantly increased in diabetic vascular tissues.[29] However, other have found no effect[30] or decreased iNOS expression in vascular smooth muscle cells (VSMC).[31] As reported by Pacheco et al.[32] high glucose levels increase the induction of iNOS and the subsequent NO production, through the activation of the PKC-βII isoform in VSMC from normotensive but not from hypertensive rats.

Normally tetrahydrobiopterin (BH_4) is a cofactor of NO synthase (NOS) that is required for NO production from endothelial NOS (eNOS) and neuronal NOS (nNOS). A lack of BH_4 leads to eNOS uncoupling and then generation of superoxide anion ($O_2^{\cdot-}$), by a mechanism generally attributed to the oxidation of BH_4 to BH_2 by $ONOO^-$ formed from the near diffusion rate reaction between $O_2^{\cdot-}$ and NO. This accumulation of BH_2 is sufficient to displace BH_4 from eNOS, resulting in uncoupling of the enzyme at the heme group within the oxygenase domain.[33] Several studies suggest that diabetes induces alterations of calcium handling and impairs NO formation in coronary endothelial cells, due to BH_4 deficiency, and in the contractile proteins that limit myocardial function independent of altered metabolism.[34,35] According to the present results, it can be deduced that *M. oleifera* extract inhibits the uncoupling of NOS activity and iNOS up-expression induced by hyperglycemia (Table 4).

The paraoxonase (PON1) is the most studied enzyme of the family. It is synthesized primarily in the liver and appears mainly in serum, where is a mostly bound to high-density lipoprotein particle (HDL), that has antioxidant and anti-inflammatory activities.[36] While that PON2 is located intracellular and PON3, although appears also in serum, is around 2 orders of magnitude less abundant that PON1.[37] An inverse relationship between PON1 activity and inflammatory responses has been described in numerous experimental models and clinical conditions, such as cardiovascular disease,[38] diabetes,[39] hypercholesterolemia,[40] and parasite infection.[41] Therefore, low levels of PON1 have been associated with the development of several pathological conditions, whereas high levels have been shown to be anti-atherosclerotic in mouse models. To our best knowledge, no information concerning effect of *M. oleifera* on PON1 activity has been published. Hence, it can be deduced that major constituents of *M. oleifera* leaves extract are capable of binding to PON1 leads to increase in maximal velocity of the reaction and/or a decrease in substrate affinity. Thus, our results tend to support a previous study by Atrahimovich et al.[42] which shown that the polyphenols, such as quercetin, bind to an allosteric site on recombinant PON1 and affect the enzyme function and biology.

Taken together, we proposed a model to explain our results. The alloxan administration causes hyperglycemia that provokes an osmotic stress on red cells causes' hemolysis and hemoglobin release. Posteriorly, high glucose reacts with Hemoglobin and other plasma protein to increase the formation of Advance glycated products may induce inflammatory process activate pro-oxidant enzymes as iNOS and NADPH oxidases (NOX2). These enzymes (iNOS and NOX2) produce ROS and RNS that damage proteins as PON 1 and uncoupling eNOS due low BH_4 levels. Thus, excessive ROS production would lead to endothelial dysfunction and cardiovascular complication Furthermore, a previous research of our group has reported that *M. oleifera* extract exhibited potent anti-hemolytic action in a dose dependent way and the results of this study indicated that extract have a great potential to prevent both HbA1C and AGEs formation caused by hyperglycemia (Figure 1).

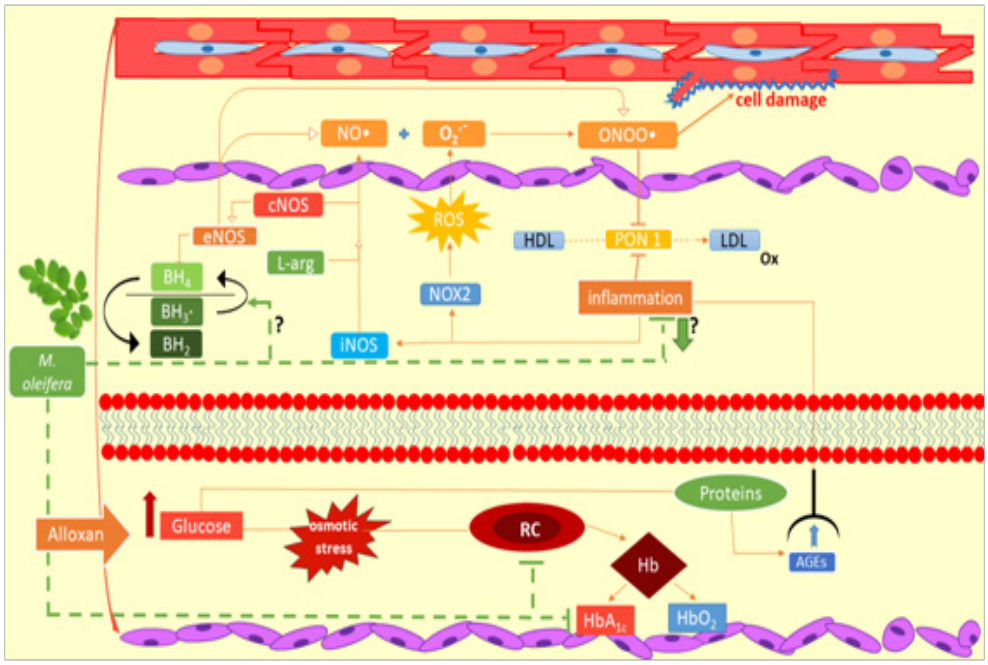

Figure 1 Proposed model for effect of *M. oleifera* extract on nitric oxide homeostasis in diabetic conditions. Hyperglycemia generated by Alloxan leads to increased AGEs pathway followed by NADPH oxidase and iNOS up-regulation. Accordingly, $ONOO^-$ formed by the NO/$O_2^{\cdot-}$ reaction could then oxidize BH_4 and therefore promote eNOS uncoupling. In addition, serum PON1 was inactivated under oxidative stress, increasing the oxidized lipid accumulation and foam cell formation. *M. oleifera* leaves extract have protective effects on NO signaling, serving as antioxidants and potential inhibitors of prooxidant enzymes as NOX2 and iNOS and may be reduce macrophage foam cell formation and atherosclerosis development.

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Conclusion

This study showed that experimental diabetes alters the homeostasis of nitric oxide by increasing the activity of iNOS and that the extract of *M. oleifera* can reverse oxidative damage because it protects PON1 and the constitutive NOS activities. However, full understanding of molecular interactions of active components of *M. oleifera* with biological targets is required to develop a new generation of antidiabetic agents for reduction or elimination of diabetic complications.

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Conflicts of interest

The authors declare no conflict of interest.

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