The nephroprotective effects of ginkgo biloba extract (EGb761) against l-N^G-nitroarginine methyl ester-induced hypertension in rats: role of oxidative stress and inflammatory markers

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Background
Ginkgo biloba extract 761 (EGb761) was studied for its nephroprotective effects in experimentally induced hypertension. Hypertension is increasingly a cause of end-stage renal diseases. Increased cytokine release and oxidative stress are mechanisms that appear to be involved in the pathogenesis of hypertensive renal damage. EGb has antioxidant and anti-inflammatory effects and can attenuate hypertensive renal damage.

Methods and results
Male adult Wistar rats were used in this study. Hypertension was induced in these rats by administering l-N^G-nitroarginine methyl ester (l-NAME) (10 mg/kg/day, intraperitoneal) for 12 weeks. Another group of rats received l-NAME and EGb761 (100 mg/kg/day, orally) starting from the ninth week to the end of treatment. It was found that the blood pressure was reduced at the end of 12th week in rats treated with EGb761 compared with l-NAME-treated (hypertensive) rats. EGb761-treated rats showed lower renal tissue malondialdehyde level and renal tissue tumor necrosis factor-α level when compared with l-NAME-treated rats (hypertensive).

Conclusion
EGb761 has antihypertensive effect; it can protect the kidney from hypertension through the reduction of renal inflammation and oxidative stress.

Keywords:
experimental hypertension, ginkgo biloba extract, kidney, rat, tumor necrosis factor-α

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Introduction

Hypertension is an important worldwide public-health challenge, which can lead to severe complications and target organ damage [1,2]. Hypertension is a multifactorial trait that results from the net effect of environmental and genetic factors. It has been suggested that oxidative stress plays an important role in the pathogenesis of hypertension [3,4]. Reactive oxygen species (ROS) have an important role in the homeostasis of the vascular wall; hence, they could contribute to the mechanism of hypertension [5]. Endothelial dysfunction due to increased oxidative stress is characteristic of human hypertension [6]. Furthermore, decreased total antioxidant status level was found in patients with sustained arterial hypertension [7]. Thus, hypertension was demonstrated to increase ROS production and decrease antioxidant capacity, leading to increased oxidative stress that may adversely affect vascular function [8].

Low-grade inflammation has been recognized to play a crucial pathophysiological role in hypertension and in cardiovascular disease. Inflammation participates in many processes that contribute to the development of elevated blood pressure (BP). Inflammation is involved in many hypertensive models, such as salt-sensitive hypertension. Thus, increased adhesion molecule expression, immune cell activation and infiltration, cytokine release, and oxidative stress formation are mechanisms that appear to be activated in hypertension [9,10].

In addition, Bautista *et al.* [11] and Kuklinska *et al.* [12] found that the plasma concentrations of the sensitive indicator of inflammation, high-sensitivity C-reactive protein (hs-CRP), are higher in patients with hypertension than in normotensive healthy controls. Moreover, a positive relationship between increased serum levels of hs-CRP and the risk for development of hypertension was reported [10,13,14]. Furthermore, a growing body of evidence indicates that vascular inflammation may be involved in both the initiation and development of hypertension [15].

It has been found that an intracellular adhesion molecule-1, alone or in conjunction with other adhesion molecules such as soluble E-selectin, was

confirmed as a sensitive marker of inflammatory vascular activation [16].

Hypertensive patients are reported to have high circulating levels of the proinflammatory cytokines (PICs). Moreover, these inflammatory markers are elevated in patients with target organ damage and not in uncomplicated hypertension [17].

PICs such as tumor necrosis factor-α (TNF-α) [18], interleukin-1 (IL-1) [19], and IL-6 [20] have been reported to increase with the severity of hypertension and are of prognostic significance. More importantly, PICs have been found to activate ROS, which in turn can activate various intracellular signaling pathways including that of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). Activation of NF-κB induces gene transcription of PICs, which leads to a further increase in ROS production, fostering a positive feedback mechanism and eventually leading to the progression of hypertension [21,22]. It has been suggested that NF-κB blockade reduces cytosolic and mitochondrial oxidative stress and attenuates hypertension in spontaneously hypertensive rats (SHRs) [23]. It was found that reduced NF-κB activity was also associated with reduced PICs and oxidative stress [24].

Hypertension causes target organ damage that involves the vasculature, heart, brain, and kidneys. Complex biochemical, hormonal, and hemodynamic mechanisms are involved in the pathogenesis of target organ damage. Common to all these processes is an increased bioavailability of ROS. Both in-vitro and in-vivo studies explored the role of mitochondrial oxidative stress as a mechanism involved in the pathogenesis of target organ damage in hypertension, especially focusing on atherosclerosis, heart disease, renal failure, and cerebrovascular disease [25].

Several experimental models for producing renal failure from hypertension have been developed. Among them, aging SHRs naturally develop Chronic kidney disease (CKD), which mimics patients with essential hypertension. When the nitric oxide synthase (NOS) inhibitor l-N^G-nitroarginine methyl ester (l-NAME) was administered to adult SHRs, the rats exhibited glomerulosclerosis and renal failure, which are similar to those observed in aging SHRs [26].

In experimental animals, several forms of hypertension were found to induce infiltration of monocytes/macrophages into the vessel wall and in target organs such as the kidney and the heart [27,28]. The role of various inflammatory mechanisms, including mononuclear leukocyte infiltration, for the development of vascular damage in atherosclerosis has been increasingly acknowledged in recent years. Complications often associated with hypertensive nephropathy include glomerular damage, resulting in inflammatory responses and compromised kidney function. Fibrosis of the tubulointerstitium develops in patients and animals with hypertension and impairs renal function, leading to organ failure [29].

It has been reported that hypercholesterolemia increases ROS production and decreases antioxidant capacity, leading to increased oxidative stress that may adversely affect vascular function [8,30]. ROS has been implicated in the initiation and progression of atherosclerosis. ROS can oxidize lipoproteins, limit the vascular availability of antiatherosclerotic nitric oxide (NO), and promote vascular expression of cytokines and adhesion molecules [8]. Moreover, it has been reported that hypercholesterolemia impairs endothelium-dependent vasodilatation due to the impairment of NO-dependent endothelial function [31]. It is now proven that inflammation plays a pivotal role in the development of atherosclerotic changes and that hypercholesterolemia can initiate and enhance the inflammatory response [32].

Ginkgo biloba extract (EGb) is an herbal dietary supplement commonly used for the treatment and prevention of aging-related cognitive decline and dementia [33]. Extracts from the leaves of ginkgo biloba, a widely planted Chinese tree, have been utilized therapeutically for decades with a uniquely broad spectrum of activity [34]. A standardized preparation of extract EGb761 has proved to be useful in cerebral and peripheral vascular insufficiency states where the pathophysiology involves free radicals and platelet-activating factor-related abnormalities. EGb761 and its constituents, especially terpenoids and flavonoids, have been reported to possess vasorelaxant properties (Duarte *et al.*, 2001), suggesting that the extract may possess protective effects against cardiovascular disease. Several studies also suggest a cardioprotective effect of ginkgo biloba through its antioxidant, antiplatelet, antithrombotic, and vasodilatory properties [35]. Furthermore, ginkgo biloba may have significant antihypertensive properties as well, providing a possible alternative mechanism for cardiovascular disease prevention. In hypertensive rats, treatment with ginkgo biloba attenuated the rise in BP [36]. In addition, a hypolipidemic action of the extract was demonstrated in rats [37]. Xie *et al.* [38] also reported that the extract inhibits the activity of HMG-CoA reductase.

EGb can diminish cytokine-stimulated endothelial adhesiveness by downregulating intracellular ROS

formation, NF-κB and activator protein-1 activation, and adhesion molecule expression in human aortic endothelial cells. It also inhibits the production of PICs, IL-1β and TNF-α, but upregulates the production of anti-inflammatory cytokines [39,40].

Materials and Methods

Drugs and chemicals

The standardized preparation of Ginkgo biloba leaf extract, EGb761, was kindly provided as a finely dispersible powder by Amriya Pharm Amriya Pharm. Ind. (Alexandria, Egypt). It was prepared as 4% suspension in 25% carboxymethylcellulose. l-NAME was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and prepared as 1% solution in saline. Malondialdehyde (MDA) bis-(dimethylacetal) was purchased from Merck (Darmstadt, Germany). Thiobarbituric acid was purchased from MP Biomedicals Inc. (Illkirch-Graffenstaden, France). Other chemicals were supplied by our Department of Pharmacology, Assiut University.

Animal groups and experimental design

Male adult Wistar rats weighing 180–200 g, obtained from animal house of Faculty of Medicine, Assiut University were used in all experiments. The animals were housed in stainless steel cages under a 12 h light/dark cycle at 25°C. The animals were allowed water *ad libitum*. The animals were classified into three groups of eight animals each: group 1 (control normal rats), which received vehicle (carboxymethylcellulose); group 2 (hypertensive rats), which received l-NAME at a dose 10 mg/kg (1% solution in saline) intraperitoneally for 12 weeks [41,42]; and group 3, which included animals that received l-NAME for 12 weeks, and, at the beginning of the ninth week, the animals received EGb at a dose of 100 mg/kg orally; it was prepared as 4% suspension in 25% carboxymethylcellulose.

Measurement of blood pressure

BP is measured with a noninvasive technique using LE5001. Noninvasive Blood Pressure Meter (Panlab Harvard Apparatus, Barcelona, Spain) provides an easy and reliable technique to measure systemic BP and cardiovascular parameters in rodents without any invasive catheterization. The animals were allowed into a restrainer, to which they were familiarized at the beginning of the experiment, and kept in a chamber at 30°C for 15–30 min to ensure reproducible BP measurements (three to four measurements/animal/session). BP was measured at the end of each week during the 12 weeks of experimental duration. All BP measurements were carried out at the same time of day of measurement.

Preparation of kidney homogenates for biochemical measurements

At the end of the experiment, the animals were killed. The blood was collected and both kidneys were isolated. One kidney was kept in formalin 10% for histopathological and immunohistochemistry studies. The other kidney was dissected and cleaned from fat and other tissues, and then cut into small pieces and weighed. Thereafter, the sample was homogenized in saline and centrifuged (10000 rpm) for 10 min. The supernatant was separated and kept in −20°C for biochemical studies.

Determination of renal malondialdehyde level

MDA is an end product of lipid peroxidation, and it was determined with the use of the thiobarbituric acid reactive substance method previously described by Ohkawa *et al.* (1979). MDA forms a 1:2 adduct with thiobarbituric acid, which can be measured spectrophotometrically.

Determination of renal tumor necrosis factor-α level

The kidney level of TNF-α was determined in tissue homogenate using enzyme-linked immunosorbent assay with a commercially available enzyme-linked immunosorbent assay kit (Koma Biotech Inc., Seoul, South Korea). The procedure was carried out according to the instructions of the manufacturer.

Statistical analysis

All data were expressed as the mean ± SEM. Intergroup comparisons were made using Student's *t*-test or one-way analysis of variance. A *P* value less than 0.05 was considered statistically significant.

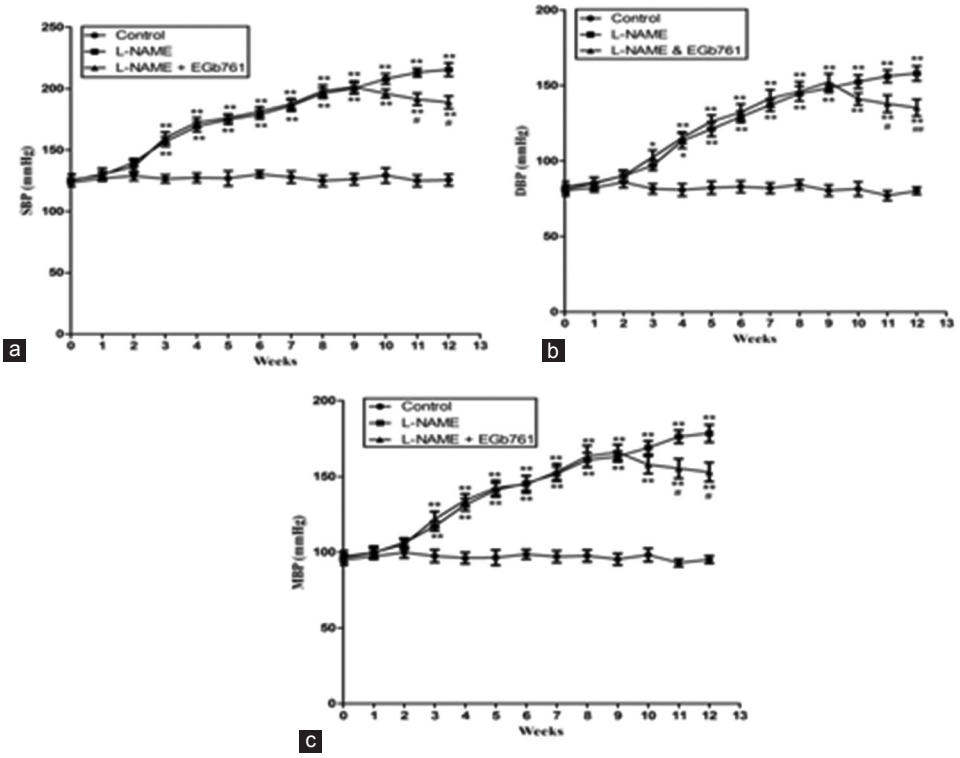
Results

Effect of l-N^G-nitroarginine methyl ester on the mean blood pressure of rats

Before treatment, the systolic BP was 123.30 ± 2.95 and 125.60 ± 5.04 mmHg for the control group and the l-NAME-treated group, respectively. Administration of l-NAME alone increased the systolic BP of rats significantly from 125.60 ± 4.74 to 156.80 ± 3.83 mmHg ($P < 0.01$) after the third week of administration, to 196.10 ± 3.99 mmHg ($P < 0.01$) after the eighth week of administration, and reached 215.50 ± 5.43 ($P < 0.01$) after the 12th week of administration (Fig. 1a and Table 1).

l-NAME alone increased the diastolic BP significantly from 80.25 ± 2.43 to 97.13 ± 3.29 mmHg ($P < 0.01$) after the third week of administration, to 144.60 ± 4.92 mmHg ($P < 0.01$) after the eighth week of administration, and to

Figure 1



Effect of l-N^G-nitroarginine methyl ester (l-NAME) and ginkgo biloba extract 761 (EGb761) on (a) systolic blood pressure, (b) diastolic blood pressure, and (c) mean blood pressure. Results are presented as mean ± SEM (n = 8). **Significantly different from control (P<0.01). #Significantly different from l-NAME group (P<0.05).

Table 1 Effect of l-N^G-nitroarginine methyl ester and ginkgo biloba extract 761 on arterial blood pressure in rats (mmHg) after 12 weeks of treatment

	Control	l-NAME	l-NAME + EGb761
SBP	125.60±4.74	215.50±5.43**	188.80±5.14#
DBP	80.25±2.43	158.10±4.87**	135.30±5.59#
MBP	95.13±2.48	178.40±5.79**	152.90±6.38#

DBP, diastolic blood pressure; EGb, ginkgo biloba extract 761; l-NAME, l-N^G-nitroarginine methyl ester; MBP, mean blood pressure; SBP, systolic blood pressure. Results are expressed as means±SEM (n=8). Blood pressure was measured weekly. **Significantly different from control (P<0.01). #Significantly different from the l-NAME group (P<0.05).

158.10 ± 4.87 mmHg (P < 0.01) after the 12th week of administration (Fig. 1b and Table 1).

Before treatment, the mean pressure values were 95.13 ± 2.48 and 96.13 ± 3.08 mmHg for the control group and the l-NAME-treated group, respectively. Administration of l-NAME increased the mean arterial BP significantly from 96.13 ± 3.08 to 117.30 ± 3.14 mmHg (P < 0.01) after the third week of administration, to 160.90 ± 4.75 mmHg (P < 0.01) after the eighth week of administration, and to 178.40 ± 5.79 mmHg (P < 0.01) after the 12th week of administration (Fig. 1c and Table 1).

Effect of treatment with ginkgo biloba extract 761 on the mean blood pressure in l-N^G-nitroarginine methyl ester-treated rats

In order to link the effect of EGb761 on the BP changes to an enhanced NO release, the animals were pretreated with the nonspecific NOS inhibitor (i.e., l-NAME), and the noninvasive BP measurements were performed. EGb761 was administered at a dose of 100 mg/kg/day after 8 weeks of l-NAME administration to the end of 12 weeks of treatment.

EGb761 reduced the systolic BP at the end of 12th week to 188.80 ± 5.14 mmHg compared with 215.50 ± 5.42 mmHg in the l-NAME-treated group (P < 0.05). Diastolic BP reduced after the 12th week to 135.30 ± 5.59 in ginkgo biloba-treated rats compared with 158.10 ± 4.87 in l-NAME-treated rats (P < 0.05). The mean arterial BP was 152.90 ± 6.38 in ginkgo biloba-treated rats compared with 178.40 ± 5.79 mmHg in l-NAME-treated rats (P < 0.05) (Fig. 1 and Table 1).

Effect of ginkgo biloba extract 761 on biochemical determinations

Lipid peroxides (malondialdehyde) level

When renal MDA, a product of lipid peroxidation, was measured after administration of l-NAME

(10 mg/kg/day, intraperitoneally) for 12 weeks, it resulted in a significant increase in the level of renal tissue MDA (1152.00 ± 59.91 nmol/g wet weight) compared with the control value (359.30 ± 25.26 nmol/g w wt, $P < 0.01$) (Fig. 2). l-NAME administration for 12 weeks produced a significant increase in the level of renal tissue TNF-α from control value of 23.24 ± 1.49 to 94.44 ± 5.87 ng/g wet weight ($P < 0.01$) (Fig. 3).

Oral administration of EGb761 (100 mg/kg/day for 4 weeks) after 8 weeks of l-NAME administration lowered renal tissue MDA level to 728.00 ± 31.62 nmol/g wet weight compared with the renal tissue MDA level in rats that received l-NAME alone (1152.00 ± 59.91 nmol/g wet weight, $P < 0.01$) (Fig. 2). Moreover, EGb761 administration lowered renal tissue TNF-α to 62.53 ± 3.41 ng/g wet weight compared with rats treated with l-NAME alone (94.44 ± 5.87 ng/g wet weight, $P < 0.01$) (Fig. 3).

Discussion

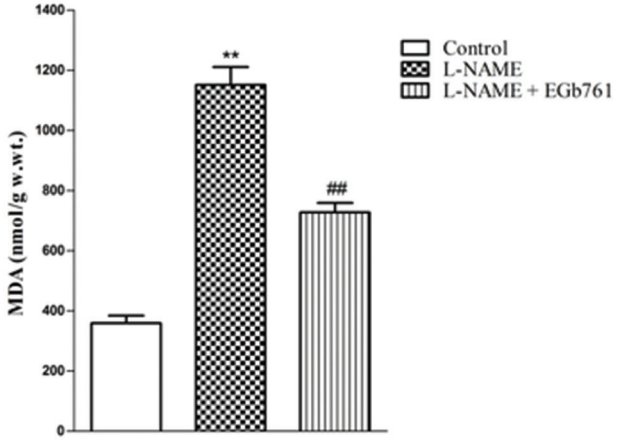
The antihypertensive effect of Gingko biloba in different animal models has been reported by several authors [43,44]. This was confirmed by the present findings, in which EGb761 produced reduction in the BP of hypertensive rats with the used dose level (100 mg/kg/day for 4 weeks).

One of the possible mechanisms that have been proposed to explain the antihypertensive effect of the extract is its inhibitory activity on ACE [45,46]. A further possible mechanism to explain the antihypertensive effect of EGb761 may be related to its pronounced antioxidant activity [47,48]. Oxidative stress is known to play an important role in establishing and maintaining high BP levels. The results of the present study demonstrated that hypertension showed variable alterations in different parameters for oxidative stress. There was a marked increase in lipid peroxidation measured as MDA in the kidneys, possibly indicating significant damage as a result of hypertension. We found that treatment with EGb reduced the MDA level the kidney.

In the present study, a significant increase in the level of renal ROS production in the hypertensive rats indicated that oxidative damage was implicated in the nephropathy caused by hypertension. The rat hypertension model induced by chronic inhibition of NO generation has been shown to be a useful tool for studying both the development and treatment of renal lesions resembling those found in human hypertension, a disease associated with early generalized impairment of endothelial function [49]. This hypertension and renal disease model seems to be a suitable one to examine the lipid-lowering-independent effects of statins because the administration of statins, even at high doses, does not alter serum cholesterol levels in normal rats [50,51].

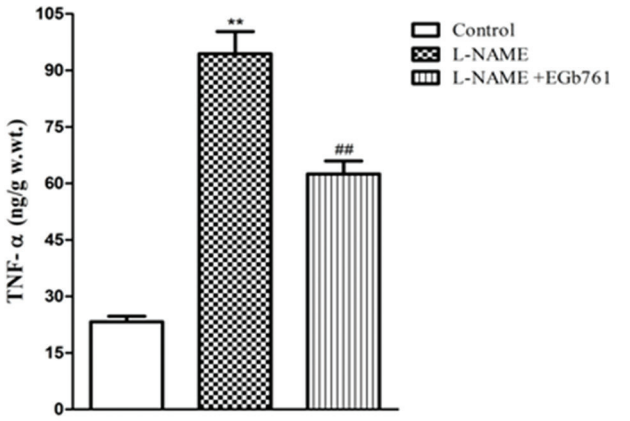
Biochemical markers, particularly markers of vascular inflammation, such as hs-CRP, have been suggested to be predictive of cardiovascular events [52]. The primary PICs TNF-α and IL-6 are the main inducers of hs-CRP. Furthermore, according to Navarro and Mora-Fernández [53], TNF-α and IL-6 have been associated with significant direct renal effects. Experimental and clinical studies have demonstrated the pathogenic role of TNF-α in the development of

Figure 2



Effect of l-N^G-nitroarginine methyl ester (l-NAME) and ginkgo biloba extract 761 (EGb761) on the level of renal malondialdehyde (MDA) level (nmol/g wet weight). Results are presented as mean ± SEM. **Significantly different from control ($P<0.01$). ##Significantly different from l-NAME group ($P<0.01$).

Figure 3



Effect of l-N^G-nitroarginine methyl ester (l-NAME) and ginkgo biloba extract 761 (EGb761) on the level of renal tumor necrosis factor-α (TNF-α) level (ng/g wet weight). Results are presented as mean ± SEM. **Significantly different from control ($P<0.01$). ##Significantly different from the l-NAME group ($P<0.01$).

renal injury and the potential benefit of modulating TNF-α activity as a therapeutic target in diverse renal diseases [54].

The 2-kidney, 1-clip model of hypertension was used by Mansour *et al.* [55] to investigate the potential antihypertensive effect of a standardized leaf extract of Ginkgo biloba (EGb761). Treatment of hypertensive rats with EGb761 orally led to a dose-dependent reduction in systolic blood pressure, with no significant change in heart rate. Mansour *et al.* [55] also found that the MDA level was raised neither in clipped kidneys or in nonclipped ones nor in the serum by treatment with EGb761.

Xiong *et al.* [56] showed that six trials demonstrated the potential positive effect of EGb761 as complementary therapy on BP reduction when compared with antihypertensive drug therapy; however, it was not associated with a statistically significant effect on both systolic blood pressure and DBP reduction in three other trials. Despite the positive findings, there were so many methodological limitations and significant clinical heterogeneity. Most of the trials did not report adverse effects, and the safety of EGb761 is still uncertain.

Accumulating evidence suggests that ginkgo biloba is cardioprotective, in part, through its vasodilatory and antihypertensive properties. However, definitive data on its BP-lowering effects in humans are lacking. Brinkley *et al.* [57] determined the effects of EGb on BP and incident hypertension in 3069 participants from the Ginkgo Evaluation of Memory study. Data indicate that ginkgo biloba does not reduce BP or the incidence of hypertension in elderly men and women.

Systemic infusion of a NOS inhibitor, l-NAME in anesthetized mice increased plasma level of TNF-a (a PIC) and increased renal tissue expression of TNF-a protein (Yang *et al.*, 2008).

Our study found that l-NAME-induced hypertension increased renal inflammation and oxidative stress. This was demonstrated through the elevation of the renal level of TNF-α and MDA when compared with control normal rats. Administration of EGb761 to hypertensive rats for 4 weeks results in a significant reduction in BP, but not normalized to control values in comparison with l-NAME-induced hypertension. We demonstrated that EGb761 reduced renal level of TNF-α and MDA when compared with hypertensive rats. We suggest that EGb761 may act by its investigated antioxidant and anti-inflammatory actions.

In conclusion, EGb761 has mild antihypertensive activity, possibly by its antioxidant and anti-inflammatory actions. It has nephroprotective action through the reduction of inflammatory markers and oxidative stress in hypertensive rats.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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