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Research Article

VASOPROTECTIVE EFFECT OF *PHYSALIS ANGULATA* L. LEAF WATER EXTRACT ON KIDNEY OF NΩ-NITRO-L-ARGININE METHYL ESTER-INDUCED ENDOTHELIAL DYSFUNCTION RAT MODEL

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ABSTRACT

Objective: This study was to investigate the effect of *Physalis angulata* L. leaf water extract on vascular rarefaction, oxidative stress, and inflammation on the kidney of Nω-nitro-L-arginine methyl ester (L-NAME)-induced male Wistar rats.

Methods: A total of 25 male Wistar rats were divided into five equal groups (normal control: 40 mg/kg/day of normal saline; L-NAME group; and treatments I, II, and III: L-NAME plus *P. angulata* L. leaf water extract doses 500 mg/kg/day, 1500 mg/kg/day, and 2500 mg/kg/day, respectively). Endothelial dysfunction was induced by 40 mg/kg/day L-NAME intraperitoneally. The treatment lasts for 15 days. Vascular rarefaction was indicated by the decrease in vascular density, which considered as vascular number per high-power field in hematoxylin-eosin staining preparation. Kidney oxidative stress test was performed by measuring malondialdehyde (MDA) level with thiobarbituric acid reactive substances assay. The inflammatory marker was the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) expression which examined using an immunohistochemical method with an antibody against p65.

Results: At the dose of 500 mg/kg/day and 1500 mg/kg/day, *P. angulata* leaf water extract supplementation increased the vascular density, decreased the MDA level, and decreased the NF-κB expression compared to the L-NAME group.

Conclusion: The administration of *P. angulata* L. leaf water extract in particular concentration has a vasoprotective effect by preventing kidney vascular rarefaction, oxidative stress, and inflammation on L-NAME-induced male Wistar rat.

Keywords: Endothelial dysfunction, Kidney, Malondialdehyde, Nuclear factor kappa-light-chain-enhancer of activated B cells, Nω-nitro-L-arginine methyl ester, *Physalis angulata* leaf, Vasoprotective.

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INTRODUCTION

Endothelial dysfunction is indicated by the decreased bioavailability of vasodilators, primarily nitric oxide (NO) [1,2]. In the kidney, NO has various important functions, such as regulation of renal hemodynamics, regulation of glomerular microcirculation and salt balance, blunting of tubuloglomerular feedback, and modulation of renal sympathetic nerve activity [3,4]. The inhibition of NO synthesis by Nω-nitro-L-arginine methyl ester (L-NAME) in rats leads to endothelial vasoconstriction and activation indicated by pro-inflammatory, proliferative, and procoagulant conditions [4,5]. It results in severe hypertension and causes kidney damage [3,4].

Endothelial dysfunction creates an imbalance between NO and reactive oxygen species (ROS) which leads to oxidative stress [6]. ROS can attack various biomolecular components of the cells, such as lipid, which causes lipid peroxidation (LPO). This reaction released LPO products, such as malondialdehyde (MDA) [3]. ROS also activates one of the transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which will subsequently induce the synthesis of inflammatory mediators, and vice versa [7]. Excessive NF-κB activation leads to excessive inflammation and cell damage [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction indicated by the decrease in vascular density [9,10]. Kidney capillary rarefaction is associated with aging and renal fibrosis and is an indicator of impaired renal function [11]. Therefore, preventing kidney vascular rarefaction, oxidative stress, and inflammation is important in the management of endothelial dysfunction associated kidney diseases.

Ciplukan (*Physalis angulata* L.) leaf is known to have high antioxidant effects *in vitro* [12]. The previous study proved that *P. angulata* leaf extract contains *Physalin,* a class of secosteroids. This compound could increase the release of NO from endothelial cell *in vitro*. This increase of NO release is thought to occur through genomic effects by increasing the expression of endothelial NO synthase (eNOS) and inducible NO synthase and non-genomic effects by increasing cytosolic calcium [13]. Another study proved the target of *P. angulata* leaf water extract in NO synthesis pathway through increased levels of vascular endothelial growth factor (VEGF) and eNOS [14].

The objective of this study is to investigate the effects of *P. angulata* L. leaf water extract, which contains physalin, withanolides [13,15,16], and flavonoid [17] on vascular rarefaction, oxidative stress, and inflammation in the kidney of NOS-inhibited Wistar rats by L-NAME.

METHODS

Animal preparation

A total of 25 male Wistar rats, weighing 250–300 g, were placed in a quiet room with cage temperature 21±2°C, in which a 12–12 h light-dark cycle was maintained. They were fed and watered by *ad libitum*. After 7 days of acclimatization, Wistar rats were divided into five groups (control negative: normal saline 0.9%, i.p; L-NAME group: 40 mg/kg/day L-NAME i.p; and L-NAME 40 mg/kg/day, i.p. + *P. angulata* leaf water extract doses of 500, 1500, and 2500 mg/kg/day by gavage, respectively). The treatment lasts for 15 days [3,18,19]. The dose of water extract of *P. angulata* leaves was determined based on the previous study [20]. The procedures were approved by the Health Research Ethics Committee of Brawijaya University (No.114/EC/KEPK/03/2017).

Extract preparation
P. angulata leaves were obtained from Balai Penelitian Tanaman Obat dan Rempah, Lembang, Indonesia. The sample of the herbs was determined and confirmed as *P. angulata* species by the Laboratory of School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia. As much as 10 g of *P. angulata* leaves dry powder were soaked in 100 ml of boiled water for an hour. The solution was filtered from the precipitated product by cotton cloth to obtain 80 ml thick extract. The same process was repeated for the remaining product. It was soaked again in 30 ml boiled water for an hour and then filtered to obtain 20 ml extract. From this procedure, we got 100 ml *P. angulata* leaf extract with a concentration of 10% (w/v).

Determination of MDA level
Kidney LPO was used as an indicator of oxidative stress [21]. It was measured according to the concentration of thiobarbituric acid (TBA) reactive substances. The amount of produced MDA was used as an index of LPO [22]. MDA and TBA react and produce pink pigment with a maximum absorption at 535 nm [23]. Tissue homogenate (0.1 ml) was mixed with 2 ml reagent consisting of 0.37% TBA, 0.25 N HCl, and 15% trichloroacetic acid with 1:1:1 ratio. The mixture was then placed in a boiling water bath for 15 min. After cooling, the mixture was centrifuged at room temperature for 10 min and the absorbance of the clear supernatant was read at 535 nm [24]. Data were expressed as nm/300 mg tissue.

Immunohistochemical preparation and evaluation
At the end of the experimental period, the rat kidneys were removed, fixed in 10% buffered formalin solution, and then embedded in paraffin. Each kidney was cut in a sagittal section into two halves. Paraffin kidney sections (5 mm) were prepared for immunohistochemical examination, and another was stained with hematoxylin and eosin. For immunohistochemical analysis of NF-κB, antigen unmasking was performed by heating the sections in citrate buffer, pH 6.0, using a water bath 95°C for 20 min. Sections were incubated overnight with primary antibodies (1:100 polyclonal p65, Santa Cruz Biotechnology) at 4°C. The detection of immunopositive cells used the avidin-biotin-peroxidase complex method. Immunoreactivity was visualized with diaminobenzidine. Hematoxylin was used as the counterstain. Negative controls consisted of each case in which the primary antibody was omitted.

Renal sections were scored for the presence of p65 in renal cells, whether in the cytoplasm or in the nucleus. Renal cells were quantitatively measured by counting at least 20 randomly selected high-power fields (×400) in the cortex and outer medulla area. The final score obtained was expressed as the number of positive cells per high-power fields [25].

Histopathological evaluation
The vascular density of the kidneys was evaluated by counting the vascular in 20 randomly selected field sites in the renal cortex and outer medulla with a magnification of ×400. It will be considered as vascular if there is a lumen containing erythrocytes and coated by endothelial cell(s) [26]. Data were expressed as the number of vascular per high-power field.

Statistical analysis
The data were analyzed with SPSS 23.0 for Windows using independent t-test to compare the control and L-NAME group. One-way analysis of variance was used to compare the L-NAME group and the treatment groups. Differences between group were determined using *post hoc* analysis in which the significance level was described as $p<0.05$.

RESULTS

The L-NAME effect on vascular density, MDA levels, and NF-κB expression in rat kidney
The effects of L-NAME on vascular density, MDA levels, and NF-κB expression in rat kidney are shown in Fig. 1a-c, respectively. L-NAME tended to decrease the vascular density in rat kidney compared to the control group, but it was not statistically significant, while the MDA level and NF-κB expression tended to increase in the L-NAME group compared to the control group, but it was not significantly different.

The effect of *P. angulata* leaf water extract on kidney vascular density
Administration of *P. angulata* leaf water extract at dose 1 (500 mg/kg/day) and dose 2 (1500 mg/kg/day) could increase vascular number significantly to L-NAME group. Increasing the dose of *P. angulata* leaf water extract tended to decrease the vascular number in renal cortex compared to dose 1 (500 mg/kg/day). At dose 3 (2500 mg/kg/day), there was significant decrease on vascular number compared to dose 1 (500 mg/kg/day) but not significant to dose 2 (1500 mg/kg/day) (Figs. 2 and 3).

The effect of *P. angulata* leaf water extract on kidney MDA level
MDA level is an important indicator of oxidant status. The data of MDA level in L-NAME plus *P. angulata* leaf water extract with various dose groups are indicated in Fig. 4. There was a decrease in MDA level of *P. angulata* leaf water extract at dose 1 of 500 mg/kg/day compared to L-NAME group. Increasing the dose of the *P. angulata* leaf extract at dose 2 (1500 mg/kg/day) and 2500 mg/kg/day tended to increase the MDA level compared to the lowest dose (Fig. 4).

The effect of *P. angulata* leaf water extract on NF-κB p65 expression
The effect of L-NAME and *P. angulata* leaf water extract on NF-kB expression is shown in Fig. 5. It was shown that the administration of 500 mg/kg/day (dose 1) *P. angulata* leaf water extract tends to decrease p65 expression on rat kidney, but it was not significant. Administration of *P. angulata* leaf water extract at higher doses (doses 2 and 3) tends to increase p65 NF-κB expression compared to dose 1 (Figs. 5 and 6).

DISCUSSION

This study showed that the vascular density in the renal cortex was decreased after 15 days of L-NAME induction followed by the increasing level of MDA and NF-κB expression (Fig. 1). It was in accordance with the previous studies, showing that L-NAME may cause endothelial dysfunction by lowering NO which promotes thrombosis, vasospasm, vascular inflammation, and proliferation of vascular smooth muscle cells [1]. Oxidative stress also contributes to the mechanisms of endothelial dysfunction. L-NAME may increase oxidative stress by enhancing nicotinamide adenine dinucleotide phosphate oxidase expression [27,28]. Cell membranes composed of poly-unsaturated fatty acids are particularly susceptible to oxidative attack, which resulted in the changes of permeability, membrane fluidity, and cellular metabolic functions. MDA, one of the LPO products, was found to increase in oxidative stress state [21]. L-NAME can induce inflammation and kidney damage by increasing angiotensin (AT) 2 stimulation to AT1-receptor [29] so that it activates the transcription factor NF-κB [30]. These processes finally will lead to microvascular rarefaction [9,31], which was indicated by a decreased in vascular density [10].

The insignificant result of all three variables in the L-NAME group compared to the control group can be caused by a relatively short duration of treatment and/or less L-NAME dose being used. A study by Cipolla *et al.* [32] showed that it took 5 weeks of L-NAME administration at a dose of 0.5 g/L drinking water to cause loss of vascular (rarefaction) structures in the Sprague-Dawley rat brain capillaries. Meanwhile, the other studies were performed for 7–8 weeks to make significant renal damage and inflammation [4,33-36]. The other needed 28 days to significantly increase rat kidney MDA level [37-39]. These studies indicate that the kidney needs a longer duration than 15 days of L-NAME administration to significantly decrease the vascular density and increase the MDA level as well as the NF-κB expression. Kidney endurance may contribute to this phenomenon. The kidney can protect itself from L-NAME-induced hypertension by its autoregulation mechanism. This autoregulation mechanism causes kidney blood

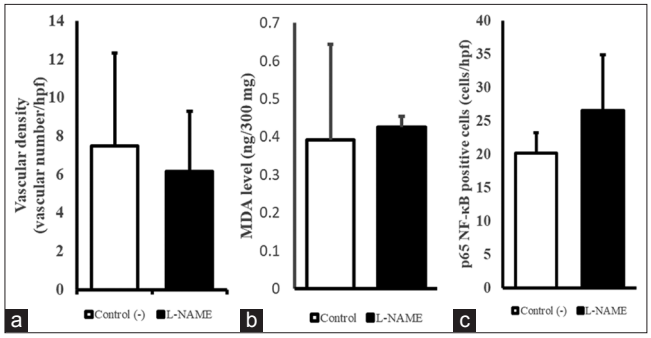

Fig. 1: Mean of vascular density (a), mean of malondialdehyde level (b), and mean of p65 expression (c) control (without Nω-nitro-L-arginine methyl ester [L-NAME]) and L-NAME (40 mg/kg/day). Data were presented as mean ± standard error of the mean and analyzed using independent t-test

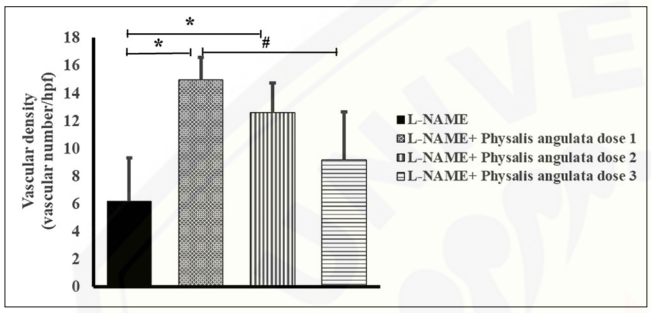

Fig. 2: Mean of kidney cortex vascular density. *p<0.05 compared to the Nω-nitro-L-arginine methyl ester (L-NAME) group. #p<0.05 compared to L-NAME + *Physalis angulata* dose 3. Data were analyzed using analysis of variance and *post hoc* test

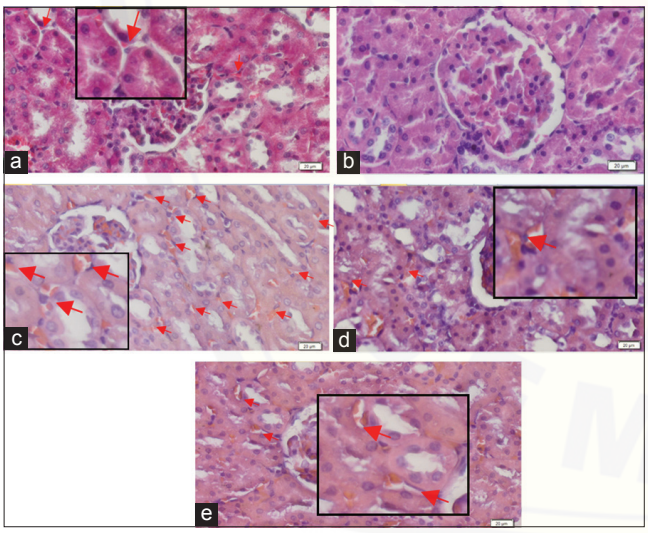

Fig. 3: Kidney cortex of negative control group (a), Nω-nitro-L-arginine methyl ester (L-NAME) group (b), L-NAME+extract dose 1 group (c), L-NAME + extract dose 2 group (d), L-NAME + extract dose 3 group (e). The red arrow indicates vascular. HE, ×400, inset ×1000, scale bar: 20 µm

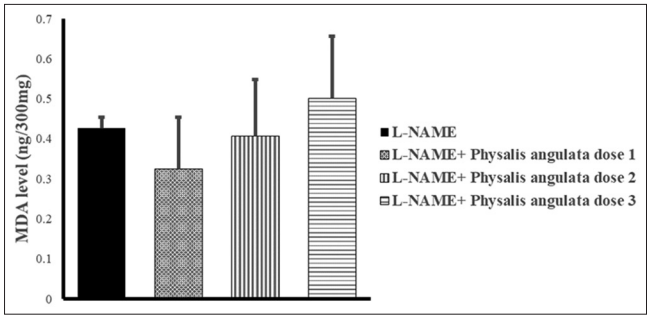

Fig. 4: Mean of kidney malondialdehyde level. Data were analyzed using the analysis of variance test and *post hoc* test

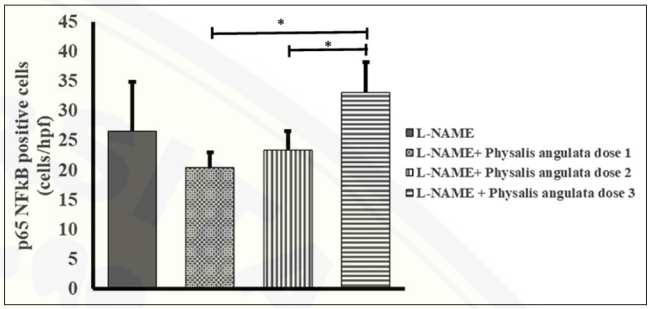

Fig. 5: Mean of p65-positive renal cells. Data were analyzed using the analysis of variance test and *post hoc* test. *p<0.05 compared to Nω-nitro-L-arginine methyl ester + dose 3 group

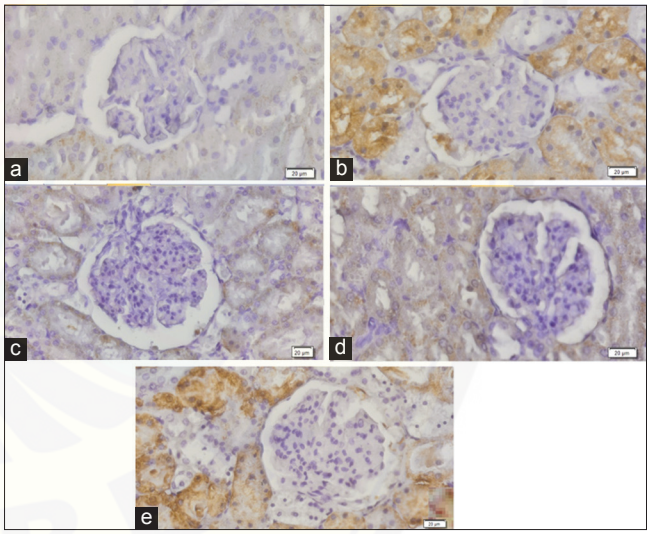

Fig. 6: Kidney p65 nuclear factor kappa light chain enhancer of activated B cells expression of the negative control group (a), Nω-nitro-L-arginine methyl ester (L-NAME) group (b), and L-NAME + extract dose 1 group (c), L-NAME + extract dose 2 group (d), L-NAME + extract dose 3 group (e). IHC, ×400, scale bar: 20 µm

pressure to be maintained normally despite an increase in systemic blood pressure [40].

Moreover, kidney cells can protect themselves from oxidative stress by synthesizing antioxidant enzymes. A study mentioned that renal ischemia would increase renal antioxidant enzymes [41]. This study showed that MDA levels in the L-NAME group did not increase significantly at the end of the treatment (Fig. 1b). It indicates that the antioxidant enzymes can still compensate for the free radical generated by L-NAME induction. Furthermore, AT2 also plays a role in kidney endurance [42]. The balance of AT2 effects on AT1 and AT2 receptors has an important impact on inducing kidney injury due to L-NAME administration. AT2 stimulation to AT1-receptor induces pro-oxidant and pro-inflammatory effects [43]. Meanwhile, AT2 stimulation to AT2-receptor counteracts those effects [44]. AT2-receptor stimulation can increase NO levels [45], possibly through direct stimulation of NOS

and bradykinin pathway [46]. The AT2-receptor stimulation reduces inflammatory response through JAK/STAT inhibition, NF-κB inhibition, and COX2 synthesis inhibition [47]. Therefore, in this study, we assumed that the reduction of NO levels induced by L-NAME still can be compensated by AT2 stimulation to AT2-receptor. Thus, the vascular density in the L-NAME group decreases insignificantly, following MDA level and NF-κB expression which increase insignificantly when compared to the control group (Fig. 1a-c).

Ciplukan (*P. angulata)* was reported as an important herbal medicine in the Indian Traditional System of Medicine [15,16]. Qualitative analysis of the content of *P. angulata* leaf water extract and ethanol extract found the presence of flavonoids, saponins, terpenoids, polyphenols, tannins, alkaloids, and steroids [48,49]. Our result showed that the treatment groups receiving 500 mg/kg/day and 1500 mg/kg/day *P. angulata* leaf water extract had significantly higher vascular density compared to the L-NAME group (Figs. 2 and 3). Therefore, it can be assumed that supplementation of *P. angulata* leaf water extract can prevent L-NAME-induced vascular rarefaction. This result is in accordance with a previous study, showing that *P. angulata* could increase NO level of endothelial cell culture [13]. NO has a vasoprotective effect by preventing endothelial cell apoptosis [50] and by inhibiting caspase through S-nitrosylation of cysteine residues [51]. Adequate NO level promotes neovascularization, one of which through the VEGF pathway and fibroblast growth factor [52]. NO stimulates endothelial migration by inducing endothelial cell podokinesis, increasing the expression of αvβ3, and enhancing dissolution of the extracellular matrix through the fibroblast growth factor-induced upregulation of urokinase-type plasminogen activator [53]. Furthermore, NO may suppress the production of angiostatin, an endogenous antagonist of angiogenesis [54]. Sulistyowati also proved that the effect of Ciplukan (*P. angulata* L.) water extract on NO synthesis pathway was by increasing VEGF levels [14]. VEGF was known as a substance needs to promote vascularization [52,55].

P. angulata leaf water extract has a high antioxidant effect *in vitro* [12, 16]. This might be due to the flavonoid content of *P. angulata* which are 5-Methoxy-6,7-methylenedioxyflavone and 5,6,7-trimethoxyflavone [56]. Flavonoids prevent tissue damage from free radicals through various mechanisms. First, it reacts with free radical molecules directly because the flavonoids have hydroxyl groups with high reactivity. Second, it inhibits xanthine oxidase to prevent superoxide formation when reoxygenation occurs [57]. In accordance with those previous studies, the addition of 500 mg/kg/day and 1500 mg/kg/day *P. angulata* leaf water extract was able to lower the MDA level of L-NAME-induced Wistar rat (Fig. 4). Therefore, it can be assumed that supplementation of *P. angulata* leaf water extract can prevent oxidative stress in the kidney of endothelial dysfunction Wistar rat model.

Treatment with *P. angulata* leaf water extract at a dose of 500 mg/kg/day and 1500 mg/kg/day also decreased p65 NF-κB expression (Figs. 5 and 6). The previous study reported that *P. angulata* leaves have anti-inflammatory effects through tumor necrosis factor-α (TNF-α) inhibition [16,20]. It was known that TNF-α can trigger the classic pathway of NF-κB activation, resulting in an inflammatory response. This is reinforced by the research of Grumbach *et al.* [58], stating that NO can inhibit NF-κB activation *in vitro*. NF-κB activation will be manifested as a total increase in the expression of p65 proteins of the NF-κB complex [59]. The interesting result from the immunohistochemistry analysis above is that the p65 expression is never found in glomerular cells (Fig. 6). It indicates the involvement of glomerular autoregulation mechanism which protects the glomerulus from L-NAME-induced inflammation. Despite the changes in renal perfusion pressure, the autoregulatory mechanism keeps the renal blood flow and glomerular filtration rate constant [40].

Ciplukan (*P. angulata*) leaves have a unique characteristic. At lower dose (500 and 1500 mg/kg/day), it serves as proangiogenic, antioxidant, and anti-inflammatory substance. Conversely, at high dose (2500 mg/kg/day), it showed to decrease vascular density (Fig. 2), increase MDA level (Fig. 4), and increase NF-κB expression (Fig. 5). It indicates that there was an excessive NO formation induced by high-dose administration of *P. angulata* leaf water extract. This finding was in accordance with the previous study, showing that high NO concentration could promote endothelial cell damage [60,61]. NO cytotoxic effect is related to the chemical reactivity of peroxynitrite (ONOO−) formed from NO [62]. ONOO− caused persistent activation of NF-κB [63]. Conversely, the cytoprotective action of NO is attributed to the inhibition of NF-κB-mediated gene expression which produces ubiquitous anti-inflammatory activity [62]. The molecular mechanisms underlying the proapoptotic effect of high-dose NO remain speculative. The factors determining whether endothelial cells undergo apoptosis when exposed to NO include the amounts of NO, the different redox states of NO, and the local environment that may promote the further production of cytotoxic moieties such as ONOO−. The previous study showed that NO and ONOO− can damage DNA directly. The damaged DNA triggers the p53-dependent or p53-independent apoptotic cell death pathways which furthermore activates caspases [60]. The damaged cells are not able to synthesize VEGF again [31]. Finally, the decrease in VEGF led to vascular rarefaction [64].

Moreover, exogenous antioxidants can act as a double-edged sword, becoming antioxidant at low doses and prooxidant at high doses [65,66]. Therefore, increasing *P. angulata* leaf water extract dose will probably increase the antioxidant which reacts as a prooxidant. Another study by Nnamani *et al.* [67] reported that *P. angulata* leaves contain cyanide. Cyanide can cause oxidative stress in cells resulting in cell death [68,69]. Therefore, we hypothesized that a high concentration of *P. angulata* leaf water extract has a contrary effect, which is antiangiogenic, pro-oxidant, and pro-inflammatory. We also hypothesized that the high MDA level of the treatment group receiving 2500 mg/kg/day *P. angulata* leaf water extract might be caused by the effect of cyanide which becomes more dominant.

CONCLUSION

Based on those results, we concluded that the administration of *P. angulata* L. leaf water extract in particular concentrations has a vasoprotective effect by preventing kidney vascular rarefaction, oxidative stress, and inflammation on L-NAME-induced male Wistar rat. This study implies the importance of the optimal dose of *P. angulata* leaf water extract supplementation for the prevention of endothelial dysfunction-induced kidney injury. However, vascular rarefaction pathway is not only triggered by NF-κB and oxidative stress but also triggered by several pathways such as VEGF and proapoptotic signaling for vascular rarefaction. To investigate this pathway, further studies are needed.

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AUTHOR CONTRIBUTION

All the authors have the same contribution in this research (carried out the research, collected the data, analyzed the data, and formatted the manuscript).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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