The increase of NO release is thought to occur through [12]. The previous study proved that antioxidant effects of Ciplukan (the management of endothelial dysfunction associated kidney diseases). Vascular rarefaction, oxidative stress, and inflammation is important in capillary rarefaction is associated with aging and renal fibrosis and is an indicator of renal vascular rarefaction indicated by the decrease in vascular density [9,10]. Kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefaction, which is characterized by reduced vascular density [8]. The damaged kidney vascular cells lead to structural changes called renal vascular rarefraction 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 Physalis 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 the synthesis of endothelial NO synthase (eNOS) and inducible NO synthase and non-genomic effects by increasing cytosolic calcium [13]. Another study proved the target of Physalis angulata leaf 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 Physalis angulata leaf 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.
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 field [25].

Histopathological evaluation

The vascular density of the kidneys was evaluated by counting the vascular structure 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 groups. One-way analysis of variance was used to compare the L-NAME groups 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-κB 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, vasoconstriction, 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 polyunsaturated 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
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. 1). 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.
Fig. 1a-c. P. angulata leaf water extract has a high antioxidant effect.

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 leaf extract can 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 angiotensin, an endogenous antagonist of angiogenesis [54]. Suliyanto et al. also proved that the effect of Ciplukan (P. angulata L.) water extract on NO synthesis pathway was by increasing VEGF levels [4], 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-methylenedioxylavone 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 the 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 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 an 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.

**ACKNOWLEDGMENT**

This research was supported by grants from The Ministry of Research, Technology, and Higher Education of the Republic of Indonesia through INSINAS 2016 funding program.

**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.

**REFERENCES**


