

***Physalis angulata* Leaf Ethanol Extract Reduces Oxidative Stress and Improves Endothelial Progenitor Cells in L-NAME-Induced Hypertensive Rats**

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ABSTRACT

This study aimed to evaluate the effects of ciplukan (*Physalis angulata* L.) leaf ethanol extract on L-N^G-nitroarginine methyl ester (L-NAME)-induced hypertensive rats. We randomly divided twenty-five Wistar rats into five groups. The sham group was given a PBS injection. The hypertensive group was injected with L-NAME on days 1 to 28. Three groups of hypertensive rats were given the extract on days 4 to 28. Blood pressure was measured using the tail-cuff method on days 0, 4, 10, and 27. The endothelial progenitor cells (EPCs) in the blood were measured by flow cytometry as a percentage of circulating angiogenic cells (CACs, CD34⁺/CD309⁺/CD45⁺) and endothelial colony-forming cells (ECFCs, CD34⁺/CD309⁺/CD45⁺). Serum NO and MDA levels, as well as serum SOD activity, were measured colorimetrically. Serum TNF- α levels were measured by the ELISA method. The ciplukan leaf extract reduced systolic and diastolic blood pressure, reduced the percentage of EPCs in the blood, increased serum NO levels, reduced MDA levels, increased serum SOD activity, and reduced serum TNF- α levels in L-NAME-induced hypertensive rats. It is concluded that ciplukan ethanol leaf extract exerts protective effects on L-NAME-induced hypertensive rats. These study results can strengthen the scientific basis of using ciplukan leaf ethanol extract to treat hypertension.

1. Introduction

One in three adults worldwide has hypertension, and its prevalence increases with age (World Health Organization 2013). According to the Indonesia Family Life Survey (IFLS-5) data in 2015, the prevalence of hypertension in Indonesia is 33.4%. Of these hypertensive patients, only 11.5% took antihypertensive drugs, and only 14.3% had their blood pressure controlled (Peltzer and Pengpid 2018). Poor hypertension control contributes significantly to cardiovascular disease. In Indonesia, cardiovascular disease is still the highest-burden (Abdi 2019).

Several antihypertensive drugs have been developed to lower blood pressure and the risks associated with high blood pressure (Karnes and Cooper-DeHoff 2009). However, the affordability and availability of pharmaceutical antihypertensive drugs is a significant challenge, especially for rural communities. This condition affects patient adherence to the treatment regimen (Tata *et al.* 2019). Furthermore, the side effects of pharmaceutical drugs (Benzie and Wachtel-Galor 2011) and the belief that plant-based medicines are safer contribute to the preference for plant extracts over pharmaceutical drugs (Alamgir 2017).

One natural ingredient that has the potential to lower blood pressure is the ciplukan (*Physalis* spp.) leaf extract. It has been shown that *Physalis minima*

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leaf extract promotes reendothelialization due to an increase in NO bioavailability in deoxycorticosterone acetate (DOCA)-salt-induced endothelial dysfunction in rats (Nugrahenny *et al.* 2017). In this study, we evaluated the effects of *Physalis angulata*, a more common species found in Indonesia, in a different model of endothelial dysfunction induced by L-N^G-nitroarginine methyl ester (L-NAME). We also determined its effects on hypertension. This study is needed as a scientific basis for developing *Physalis angulata* herbal products as an alternative therapy for hypertension.

2. Materials and Methods

2.1. Plant Materials and Extraction

The extract was made from dried ciplukan (*Physalis angulata* L.) leaves obtained from the Herbs and Medicines Research Institute (BALITRO), Bogor. Plant determination was carried out at the School of Life Sciences and Technology, Institut Teknologi Bandung (SITH-ITB, No. 3538/11.CO2.2/PL/2016). According to standard procedures, the extraction was performed by maceration with 96% ethanol solvent. The extraction resulted in the form of a thick dark green paste. It was put in a glass bottle and stored at -20°C until used.

2.2. Animals

The rats used in this study were obtained from the Faculty of Pharmacy, Gadjah Mada University. The inclusion criteria for rats were female Wistar rats, 10-12 weeks of age, bodyweight 160-180 grams, healthy, and had never experienced any chemical treatment and intake. The rats that died before the sacrifice time were excluded from the study.

This study received ethical approval from the Research Ethics Commission of the Faculty of Medicine, Universitas Brawijaya. Before the experiment, the rats were adapted to laboratory conditions for 14 days. The rats were put in a plastic cage. Each cage contained five rats. The cage was placed in a room (20-25°C) set for 12 hours of light/dark. The husk pads in the cage were changed two times a week. Standard rat feed and tap water were provided ad libitum.

2.3. Experimental Protocol

Rats that met the criteria were randomly divided into five groups, namely: (1) one sham group (sham) that was given phosphate-buffered saline (PBS) injection, (2) one hypertensive group (H) was given L-NAME injection, and (3) three hypertensive groups who were given ciplukan leaf extract at doses of 500 (PA1), 1500 (PA2), and 2,500 mg/kg BW/day (PA3). The dosage used was according to the results of

previous studies (Nugrahenny *et al.* 2017). The extract was dissolved with distilled water, then given orally for four weeks starting on the 4th day after L-NAME injection.

2.4. Hypertension Induction

The solution of L-NAME (Cayman Chemical, Ann Arbor, MI, USA) was prepared at a 75 mg/kg BW dose in PBS (Sigma-Aldrich, St. Louis, MO, USA) solvent just before injection. L-NAME was injected for four weeks. The injection was performed subcutaneously (s.c.) in the lower-left quadrant abdomen to avoid the rats' vital organs. The sham group was given an injection of PBS s.c. The final injection volume was not more than 0.3 ml/rat according to the abdomen's subcutaneous capacity (Shu *et al.* 2018; Yang *et al.* 2012).

2.5. Blood Pressure Measurement

Measurement of rat blood pressure was carried out indirectly using the CODA rat tail-cuff blood pressure system (Kent Scientific Corp., Torrington, CT, USA). Measurements were made before L-NAME injection, on days four and ten after L-NAME injection, and at the end of the study period. Each blood pressure measurement was carried out for 15 repetitions, then the mean systolic and diastolic blood pressure was calculated in mmHg. Blood pressure was considered high (hypertension) if it was >140/90 mmHg.

2.6. Serum and Organ Sampling

Rats were sacrificed at the end of the study period by injecting ketamine anesthesia (40 mg/kg BW) intraperitoneally (i.p.). The blood sample (2 ml) was taken from the heart and placed in a vacutainer containing 10% EDTA for isolation of mononuclear cells to analyze the number of EPCs. The rest of the blood sample was placed in a vacutainer containing clot activator gel, then centrifuged (4,000 rpm, 10 minutes, RT) to obtain serum. The serum was stored at -40°C until analysis.

2.7. Analysis of the Number of EPCs in Blood

Cells were isolated from blood samples taken from rat hearts and given EDTA anticoagulants. The antibodies used were CD34 PE (sc-7324 PE, 200 µg/mL), Flk-1 FITC (sc-393163 FITC, 200 µg/ml), and CD45 PE-Cy5/PEC5 (sc-1178 PEC5, 100 tests, 2 ml). Antibody dilution was one µg per 1 x 10⁶ cells. Immunofluorescence staining was carried out according to manufacture procedures (Santa Cruz Biotechnology, Inc., 2019). The analysis was performed using a flow cytometer. FSC-A/SSC-A scatter gates were used around the mononuclear

cell population (lymphocytes and monocytes) and recorded 100,000 gated events per tube (Hristov *et al.* 2009). The number of EPCs in the blood was calculated as a percentage (%) of circulating angiogenic cells (CACs, CD34⁺/CD309⁺/CD45⁺) and endothelial colony-forming cells (ECFCs, CD34⁺/CD309⁺/CD45⁻) (Attar *et al.* 2017; Hubel *et al.* 2011; Parsanezhad *et al.* 2015).

2.8. Analysis of Serum TNF- α Levels

Serum tumor necrosis factor (TNF)- α levels were measured by the sandwich-ELISA principle using the rat TNF- α ELISA kit. The analysis was carried out according to the kit's procedure (Elabscience Biotechnology, Inc., Houston, TX, USA).

2.9. Colorimetry

Serum NO and malondialdehyde (MDA) levels and serum superoxide dismutase (SOD) activities were measured colorimetrically using the assay kits according to the kits' procedure (Elabscience Biotechnology, Inc., Houston, TX, USA).

2.10. Statistical Analysis

We present data as mean \pm SD. The differences between groups were analyzed using a one-way analysis of variance (ANOVA). Except, two-way ANOVA analyzed the systolic and diastolic blood pressures. Tukey's multiple comparisons test then followed the analysis. All analyses were performed with GraphPad Prism 8.4.2 for Windows. The probability values of $P < 0.05$ were considered significant.

3. Results

3.1. Blood Pressure

Before treatment, there were no significant differences ($P > 0.05$) in systolic and diastolic blood pressures. With L-NAME injection, systolic and diastolic blood pressures were increased significantly ($P < 0.02$) on day four compared to the sham rats. The ciplukan leaf extract administration at a dose of 2,500 mg/kg BW reduced systolic blood pressures on days 10 and 27 significantly ($P < 0.02$ and $P < 0.05$, respectively) compared to hypertensive rats that did not receive the extract. The ciplukan leaf extract at 1,500 mg/kg BW also significantly reduced systolic blood pressures on day 27 ($P < 0.02$) compared to hypertensive rats that did not receive the extract. The ciplukan leaf extract administration at 1,500 and 2,500 mg/kg BW tended to decrease the diastolic blood pressures on days 10 and 27, although not statistically significant ($P > 0.05$) than hypertensive rats that did not receive the extract. The blood pressures are shown in Figure 1.

3.2. Percentages of Circulating Angiogenic Cells (CD34⁺/CD309⁺/CD45⁺) and Endothelial Colony-Forming Cells (CD34⁺/CD309⁺/CD45⁻)

At the end of the study period, flow cytometry analysis was performed to determine the percentages of two types of EPCs, namely CACs (CD34⁺/CD309⁺/CD45⁺) and ECFCs (CD34⁺/CD309⁺/CD45⁻) in blood. There was an increase in the percentages of CACs ($P < 0.05$) and ECFCs ($P > 0.05$) in hypertensive rats

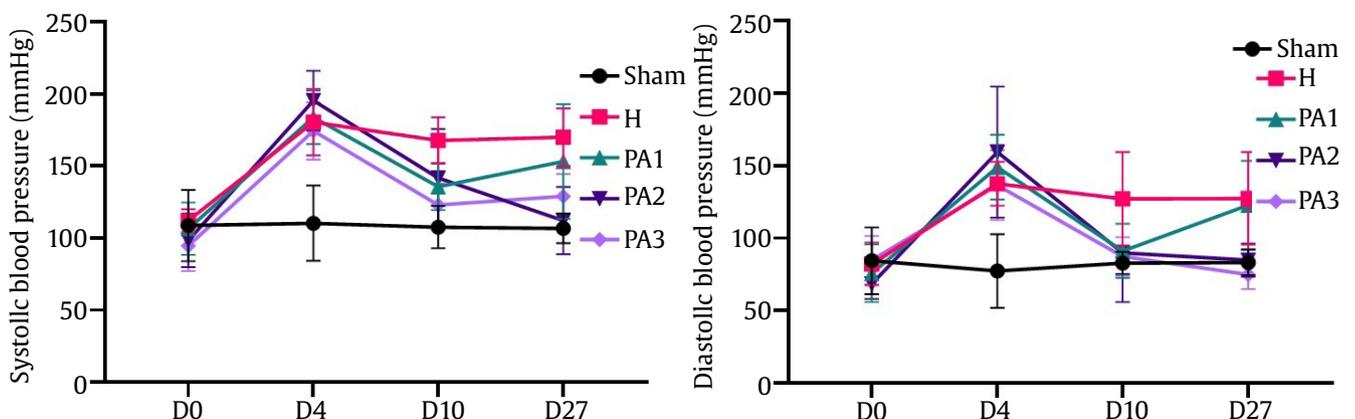


Figure 1. Rat systolic and diastolic blood pressures. D0, D4, D10, D27: blood pressures before treatment, as well as the 4th, 10th, and 27th day of treatment; H: hypertensive rats; PA1-3: hypertensive rats were given ciplukan leaves ethanol extract at doses of 500, 1,500, or 2,500 mg/kg BW/day; Sham: Sham rats. Data are presented in mean \pm SD

compared to sham rats. The ciplukan leaf extract supplementation tended to reduce the percentages of CACs and ECFCs compared to hypertensive rats that did not receive the extract, although not statistically significant ($P>0.05$). The percentages of CACs and ECFCs in the blood are shown in Figure 2.

3.3. Serum MDA Level

There was a significant ($P<0.0001$) increase in serum MDA levels in hypertensive rats than in the sham rats. Compared to hypertensive rats who did not receive the extract, the ciplukan leaf extract

administration at 1,500 and 2,500 mg/kg BW reduced serum MDA levels significantly ($P<0.01$). The serum MDA level is shown in Figure 3.

3.4. Serum SOD Activity

There was an insignificant ($P>0.05$) decrease in serum SOD activity in hypertensive rats than the sham rats. The ciplukan leaf extract supplementation increased serum SOD activity compared to hypertensive rats that did not receive the extract, with a significant result at 1,500 mg/kg BW ($P<0.05$). The serum SOD activity is shown in Figure 4.

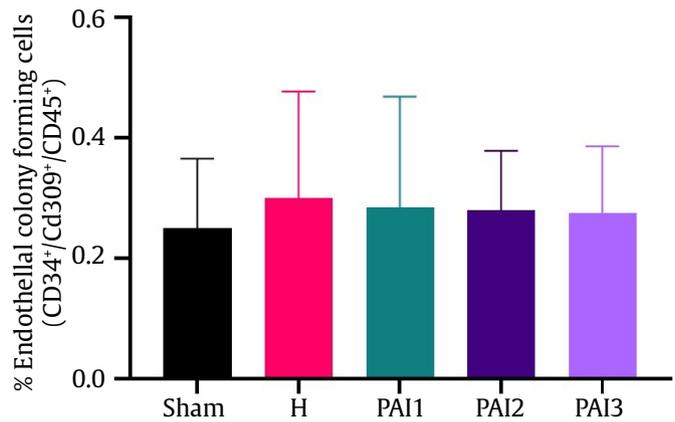
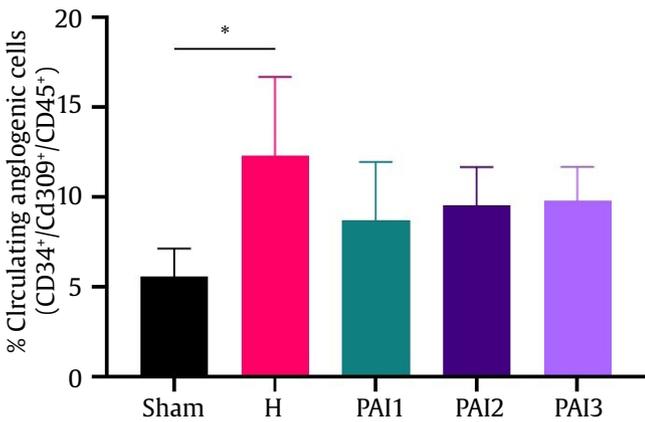


Figure 2. The percentages of CACs and ECFCs in the blood. H: hypertensive rats; PA1-3: hypertensive rats were given ciplukan leaves ethanol extract at doses of 500, 1,500, or 2,500 mg/kg BW/day; Sham: Sham rats. Data are presented in mean ± SD

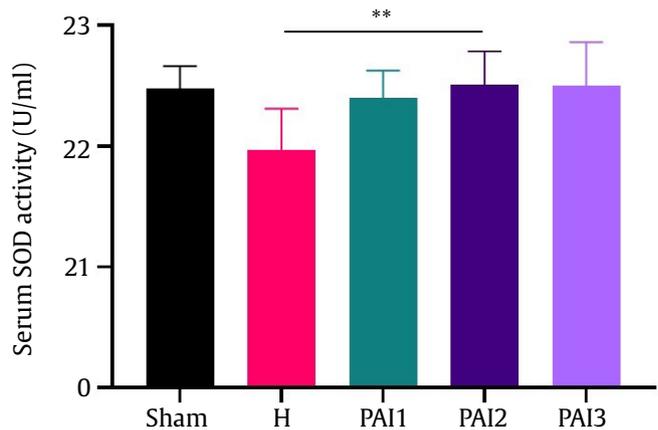
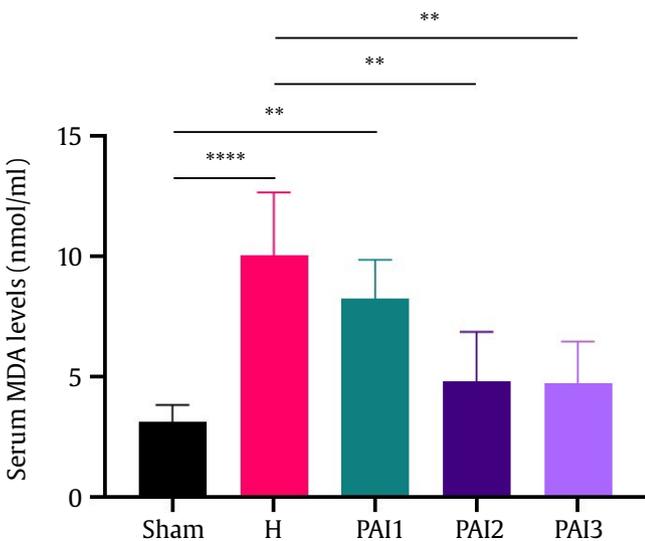


Figure 3. Serum MDA level. H: hypertensive rats; PA1-3: hypertensive rats were given ciplukan leaves ethanol extract at doses of 500, 1,500, or 2,500 mg/kg BW/day; Sham: Sham rats. Data are presented in mean ± SD

Figure 4. Serum SOD activity. H: hypertensive rats; PA1-3: hypertensive rats were given ciplukan leaves ethanol extract at doses of 500, 1,500, or 2,500 mg/kg BW/day; Sham: Sham rats. Data are presented in mean ± SD

3.5. Serum TNF- α Level

There was an increase in serum TNF- α levels in hypertensive rats compared to sham rats, although not statistically significant ($P>0.05$). Giving ciplukan leaf extract tended to reduce serum TNF- α levels compared to hypertensive rats that did not receive the extract, although not statistically significant ($P>0.05$). The serum TNF- α level is shown in Figure 5.

3.6. Serum NO Level

There was a significant ($P<0.01$) decrease in serum NO levels in hypertensive rats than the sham rats. Giving ciplukan leaf extract increased serum NO levels compared to hypertensive rats that did not receive the extract, with a significant ($P<0.02$) result at 2,500 mg/kg BW. Serum NO levels are shown in Figure 6.

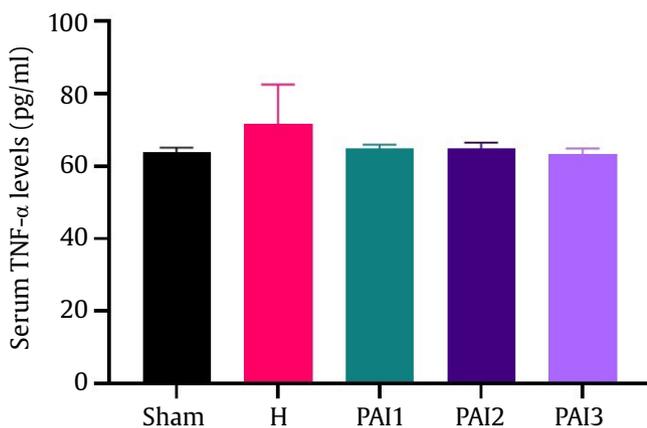


Figure 5. Serum TNF- α level. H: hypertensive rats; PAI1-3: hypertensive rats were given ciplukan leaves ethanol extract at doses of 500, 1500, or 2500 mg/kg BW/day; Sham: Sham rats. Data are presented in mean \pm SD

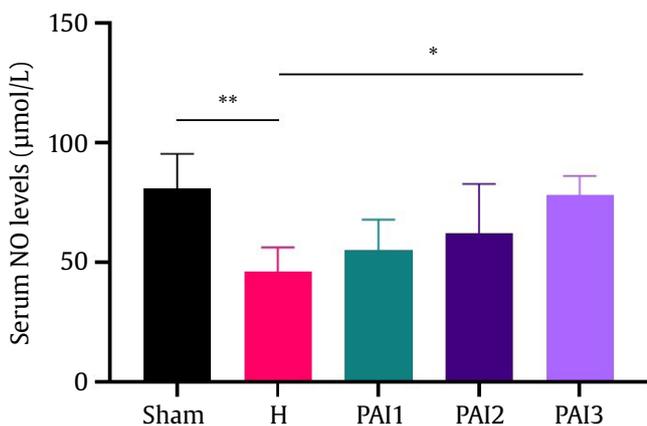


Figure 6. Serum NO level. H: hypertensive rats; PAI1-3: hypertensive rats were given ciplukan leaves ethanol extract at doses of 500, 1500, or 2500 mg/kg BW/day; Sham: Sham rats. Data are presented in mean \pm SD

4. Discussion

This study was conducted on hypertensive rats induced with L-N^G-nitroarginine methyl ester (L-NAME), an arginine derivative that competitively inhibits nitric oxide synthase (NOS). Inhibition of NOS reduces NO synthesis and impacts the damage of systemic endothelial cells, which universally underlies the pathogenesis of hypertension (Ribeiro *et al.* 1992; Vítěček *et al.* 2012). Consistent with the previous studies, L-NAME injection decreased the NO level and increased systolic and diastolic blood pressures in this study.

In this study, the level of serum TNF- α was increased in hypertensive rats. It is consistent with the previous study results that reported an increase in TNF- α in hypertension induced by L-NAME (Bunbupha *et al.* 2015; Ndisang *et al.* 2014). A high level of pro-inflammatory cytokines and the renin-angiotensin system activation induce the NADPH oxidase (NOX) system, which generates reactive oxygen species (ROS) generation (Cat *et al.* 2013).

The L-NAME injection increased serum MDA level and decreased serum SOD activity in this study. It is in line with the previous study results that showed an elevation in oxidative stress in the chronic hypertensive state (Khan *et al.* 2016). High blood pressure produces ROS due to renin-angiotensin system activation and mechanical stretch-induced vascular stimulation (Khanna *et al.* 2008; Vaziri *et al.* 2000).

In oxidative conditions, the interaction between NO and superoxide anion generates a highly toxic and reactive radical, namely peroxynitrite (ONOO⁻). Oxidative stress also induces eNOS uncoupling by decreased cofactor BH₄ and increased asymmetric dimethyl-arginine (ADMA). Peroxynitrite inactivates BH₄ by oxidating it to dihydrobiopterin (BH₂), thus responsible for eNOS uncoupling. Furthermore, the peroxynitrite induces the oxidation of LDLs, resulting in reduced L-arginine bioavailability (Channon *et al.* 2000; Chew and Watts 2004). Superoxide dismutase (SOD) exerts a defense mechanism against peroxynitrite. It reduces the superoxide into hydrogen peroxide. Then, catalase subsequently converts hydrogen peroxide to water. However, in oxidative stress, the antioxidants' defense mechanism is insufficient (Fukai and Ushio-Fukai 2011). These conditions determine an increased generation of ROS and a decreased NO synthesis and bioavailability.

A decrease in NO synthesis and bioavailability induces endothelial dysfunction in hypertensive conditions. In the early stage of endothelial dysfunction, bone-marrow-derived EPCs regenerate damaged endothelial cells. A decreased EPCs

number, a reduced EPCs mobilization, and an impaired EPCs function contribute to endothelial dysfunction progression (Higashi *et al.* 2012). In this study, there was an increased EPCs number in the blood of hypertensive rats. This study result is inconsistent with previous studies using salt-sensitive hypertensive rats (Chen *et al.* 2012; Nugrahenny *et al.* 2017). This contradiction may occur because of a difference in the animal model used. However, this study may reveal an early compensatory mechanism against endothelial damage in hypertensive conditions, promoting EPCs mobilization from the bone marrow. Although the number of EPCs increased in hypertensive rats' peripheral circulation, it is likely that they cannot repair the endothelial damage due to an impaired function, thereby promoting high blood pressure.

The present study demonstrates that treatment with ciplukan leaf extract ameliorates oxidative stress, indicated by decreased serum MDA level and increased serum SOD activity. Thus, improving the EPCs function and endothelial function, leading to reduced blood pressure in hypertensive rats induced by L-NAME. Ciplukan leaf contains flavonoids, which act as antioxidants by direct scavenging ROS, generating more stable radicals (Panche *et al.* 2016). A previous study showed that flavonoids promote NO synthesis, reduce endothelial dysfunction, and increase vasodilatation (Taubert *et al.* 2003). Ciplukan leaf extract also contains withanolide, a natural steroidal lactone (Rengifo-Salgado *et al.* 2013). A previous study revealed that a fraction of *Withania somnifera*, rich in withanolide, decreases MDA level and increases SOD, glutathione (GSH), and total antioxidant capacity (Devkar *et al.* 2016). Thus, ciplukan leaf extract reduces oxidative stress and improves NO bioavailability. These actions improve EPCs function and endothelial function, leading to reduced blood pressure..

In conclusion, As is revealed by the present study, oxidative stress can be observed in L-NAME-induced hypertensive rats, which participate in impaired EPCs function and endothelial dysfunction. These effects are partly reversed by ciplukan ethanol leaf extract treatment. It is concluded that ciplukan ethanol leaf extract exerts protective effects on L-NAME-induced hypertensive rats. The underlying pharmacological mechanism may be attributed to the improvement of EPCs function and endothelial function and the attenuation of oxidative stress, at least in part. These findings suggest that

supplementation of ciplukan leaf extract has the potential to attenuate L-NAME-induced hypertension

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Conflict of Interest

There is no conflict of interest regarding the publication of this article.

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