Sains Malaysiana 47(10)(2018): 2421–2428
http://dx.doi.org/10.17576/jsm-2018-4710-18

Antihypertensive Effect of *Piper sarmentosum* in L-NAME-Induced Hypertensive Rats

(Kesan Antihipertensi *Piper sarmentosum* pada Tikus Hipertensi Aruhan L-NAME)

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ABSTRACT

Hypertension is one of the risk factors for cardiovascular diseases and has been associated with about 13% of global deaths worldwide. Oxidative stress and reduced nitric oxide (NO) bioavailability contribute to the development of endothelial dysfunction and subsequently hypertension. N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) inhibits NO synthesis; leading to hypertension. Piper sarmentosum *(PS) is an herb with antioxidant, antiatherosclerosis and antiinflammation properties. PS also stimulated NO production by endothelial cells. The aim of this study was to determine the effects of aqueous extract of* Piper sarmentosum *(AEPS) on blood pressure, oxidative stress and the level of nitric oxide in L-NAME-induced hypertensive rats. Hypertension was induced by oral administration of L-NAME (100 mg/L) in drinking water for four weeks. The rats were concurrently treated with AEPS by oral gavage in serial doses (125, 250 and 500 mg/kg/day). Blood pressure was measured using non-invasive tail-cuff method at baseline and fortnightly thereafter. Serum level of NO and an oxidative stress marker, malondialdehyde (MDA) were measured at baseline and at the end of treatment. The results showed that treatment with three different doses of AEPS successfully reduced systolic blood pressure (*$p<0.001$*), diastolic blood pressure (*$p<0.05$*) and mean arterial pressure (*$p<0.05$*) in L-NAME-induced hypertensive rats. Treatment with AEPS also reduced MDA level (*$p<0.001$*) and increased serum NO (*$p<0.001$*) in L-NAME-induced hypertensive rats. The findings showed that AEPS decreased blood pressure by protecting against oxidative stress and increasing NO in L-NAME-induced hypertensive rats.*

Keywords: Hypertension; nitric oxide; N^{ω}-nitro-L-arginine methyl ester hydrochloride; oxidative stress; Piper sarmentosum

ABSTRAK

Hipertensi merupakan salah satu faktor risiko penyakit kardiovaskular dan ia dikaitkan dengan kira-kira 13% kematian di seluruh dunia. Stres oksidatif dan pengurangan ketersediaan biologi nitrik oksida (NO) menyumbang kepada terjadinya disfungsi endotelium yang seterusnya menyebabkan hipertensi. N^{ω}-nitro-L-arginina metil ester hidroklorida (L-NAME) merencat sintesis NO dan menyebabkan hipertensi. Piper sarmentosum *(PS) adalah herba yang mempunyai sifat antioksidan, antiaterosklerosis dan antiinflamasi. PS juga merangsang pengeluaran NO oleh sel endotelium. Tujuan kajian ini adalah untuk menentukan kesan ekstrak akueus* Piper sarmentosum *(AEPS) terhadap tekanan darah, stres oksidatif dan aras nitrik oksida dalam tikus hipertensi aruhan L-NAME. Hipertensi diaruh dengan pemberian L-NAME (100 mg/L) secara oral di dalam air minuman selama empat minggu. Dalam masa yang sama, rawatan tikus dengan AEPS turut diberi serentak melalui gavaj oral dalam dos bersiri (125, 250 dan 500 mg/kg/hari). Tekanan darah diukur menggunakan kaedah kuf ekor tidak invasif sebelum rawatan dimulakan dan setiap dua minggu selepas itu. Aras serum NO dan penanda stres oksidatif, malondialdehida (MDA) diukur sebelum uji kaji dimulakan dan selepas rawatan tamat. Keputusan menunjukkan rawatan dengan tiga dos AEPS yang berbeza berjaya menurunkan tekanan darah sistolik (*$p<0.001$*), tekanan darah diastolik (*$p<0.05$*) dan tekanan arteri purata (*$p<0.05$*) dalam tikus hipertensi aruhan L-NAME. AEPS juga menurunkan aras MDA (*$p<0.01$*) dan meningkatkan aras NO (*$p<0.001$*) dalam serum tikus hipertensi aruhan L-NAME. Keputusan yang diperoleh menunjukkan bahawa AEPS berupaya menurunkan tekanan darah dengan mengurangkan stres oksidatif dan meningkatkan aras NO pada tikus hipertensi aruhan L-NAME.*

Kata kunci: Hipertensi; nitrik oksida; N^{ω}-nitro-L-arginina metil ester hidroklorida; Piper sarmentosum*; stres oksidatif*

INTRODUCTION

Hypertension is defined as persistent elevation of systolic blood pressure of 140 mmHg or greater and/or diastolic BP of 90 mmHg or greater. According to World Health Statistic, it was reported that non-communicable diseases (NCD) were mostly caused by cardiovascular diseases (CVD) which accounted for 31% of global deaths (Mendis 2014). Hypertension is one of the risk factors for CVD and has been associated with about 17 million deaths annually. It contributes to kidney failure, stroke, heart disease and premature mortality. In the early stage, hypertension is asymptomatic and many people go undiagnosed (WHO 2012).

Endothelial dysfunction (ED) has been observed in the early stage of hypertension and it is the commonest contributing factor to hypertension (Park & Park 2015). Several studies had suggested that oxidative stress contributed to the development of hypertension through nitric oxide (NO) inactivation (Baradaran et al. 2014; Sinha & Kumar Dabla 2015). Increased free radical production as well as imbalance between the level of NO and endothelial vasoconstrictors such as endoperoxides, endothelins and thromboxane A lead to endothelial dysfunction and hypertension (Davidge et al. 2015; Panth et al. 2016; Vanhoutte et al. 2017).

Many methods to induce hypertension had been used in animal models. N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), an L-arginine analogue, acts as a competitive inhibitor of non-specific nitric oxide synthases (NOS). L-NAME has been widely used to create NO-deficient hypertensive model. It is well established that inhibition of nitric oxide biosynthesis by *in vivo* administration of L-NAME causes endothelial dysfunction and vasoconstriction, hence leading to hypertension (Raja 2010).

Piper sarmentosum is a herb that had been reported to have antioxidant activity (Hafizah et al. 2010). Toxicity studies on aqueous extract of PS showed that the extract is safe for consumption (Mohd Zainudin et al. 2013). Previous studies had reported various medicinal effects of PS such as antihyperglycemia (Azlina et al. 2009), antiatherosclerosis (Amran et al. 2010), anticarcinogenesis (Ariffin et al. 2009) and antiinflammation (Ridtitid et al. 2007; Zakaria et al. 2010). Apart from that, PS is also able to protect against glucocorticoid-induced osteoporosis (Mohamad Asri et al. 2016) and paracetamol-induced oxidative liver injury (Azlina et al. 2014). Previous studies had shown that PS is able to reduce blood pressure in spontaneously hypertensive rats (SHR) (Zainudin et al. 2015). Aqueous extract of PS is able to stimulate NO production in human umbilical vein endothelial cells (HUVECs) (Ugusman et al. 2012). This suggests that PS may regulate blood pressure via the NO pathway. Therefore, this study was designed to determine the effects of PS on blood pressure, oxidative stress and NO level in L-NAME-induced hypertensive Wistar rats.

Materials and Methods

Plant Materials

Fresh leaves of PS were collected in Selayang, Selangor, Malaysia between January to February 2012 and were identified by a plant taxonomist from the Medicinal Plant Division, Forest Research Institute of Malaysia with plant identification number (PID) 170612-11.

Preparation of Aqueous Extract of *Piper sarmentosum*

Aqueous extract of *Piper sarmentosum* was prepared following the method as reported previously (Mohd Zainudin et al. 2013). Briefly, the leaves of PS were oven-dried for 36 h at 50°C. Then the dried leaves were cut into small pieces and crushed. 10 g of dried leaves were mixed with 900 mL of distilled water. Hot water extraction was prepared by boiling the mixture at 80°C for 3 h. Then, the extract was concentrated and freeze-dried into powder form. It was stored at 4°C until use.

Experimental Animals

This study had been approved by the Animal Ethics Committee, Universiti Kebangsaan Malaysia (approval code: PP/FISIO/2011/AMINUDDIN/22-MARCH/360-JUNE-2011-JUNE-2012). Healthy adult male Wistar rats, aged 6 to 8 weeks, weighing between 170-220 g were obtained from the Laboratory Animal Resource Unit, Universiti Kebangsaan Malaysia. The rats were maintained in an air-conditioned room at 23 ± 3°C and were housed in individual cages with a 12-h light and 12-h dark cycle. The rats were provided with normal rat chow and clean drinking water *ad libitum*. The rats were acclimatized for one week before the experiment started.

Experimental Design and Induction of Hypertension

Thirty six animals were divided into six groups with six animals in each group (n=6): control group; Aqueous extract *Piper sarmentosum* (AEPS) only group where the rats were given 500 mg/kg/day AEPS via oral gavage; L-NAME-induced hypertensive group was given 100 mg/L L-NAME in drinking water and three groups of L-NAME-induced-hypertensive rats with co-treatment of different doses of AEPS; 100 mg/L L-NAME and 125 mg/kg/day AEPS; 100 mg/L L-NAME and 250 mg/kg/day AEPS; and 100 mg/L L-NAME and 500 mg/kg/day AEPS. The treatments were given for four weeks (Wheal et al. 2007). Group 2 was included in this study to investigate whether AEPS on its own could affect the parameters measured in this study. All the three doses of AEPS were adopted from previous study on anti-atherosclerosis effect of AEPS (Amran et al. 2010).

Determination of Blood Pressure

Systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) were measured in non-anaesthetized rats by non-invasive tail-cuff CODA blood pressure system (Kent Scientific Corporation, USA) at baseline and every two weeks after starting treatment (Zainudin et al. 2015). At least five readings were recorded during each measurement. The maximum and minimum values were discarded and the remaining three values were calculated as the average (Si & Liu 2008; Yang et al. 2008).

Blood Samples Collection

Blood samples (2 mL) were obtained in the morning at baseline and at the end of four weeks treatment via retro orbital sinus puncture with the animal under the

combination anaesthesia of Zoletil, Xylazil and Ketamine and were collected in plain tubes. The blood samples were centrifuged at 3000 rpm for 10 min to obtain the serum. After the separation, the serum was aspirated and was frozen at -80°C until further experiments.

DETERMINATION OF NITRIC OXIDE LEVEL

Since nitric oxide is unstable in aqueous condition and has a short half-life, the level of its stable metabolites, nitrite (NO_2^-) and nitrate (NO_3^-) was measured based on Griess method using QuantiChrom™ nitric oxide assay kit (BioAssay Systems, USA) according to the manufacturer's instructions. Total NO_2^-/NO_3^- in the serum samples were quantitated by measuring the optical density at 540 nm.

DETERMINATION OF PLASMA MALONDIALDEHYDE (MDA)

The lipid peroxidation indicator, plasma MDA was measured using thiobarbituric acid reactive substances (TBARS) assay as described previously (Borges et al. 2018). Total protein concentration in the serum was measured using protein biuret reaction (Pessoa et al. 2017). Plasma MDA level was calculated based on the following formula:

$$\frac{\text{OD sample}}{\text{OD standard}} \times \text{standard concentration} \times \frac{\text{Total volume}}{\text{volume sample} \times \text{protein (g)}}$$

Plasma level of MDA was expressed as nmol/g protein

STATISTICAL ANALYSIS

All statistical analysis was conducted using IBM SPSS Statistic version 22.0. The data were expressed as mean ± SEM. SBP, DBP and MAP results were analysed using one-way analysis of variance (ANOVA) with Tukey post-hoc test while serum nitric oxide and malondialdehyde level were analysed using paired t-test. $P < 0.05$ was considered statistically significant.

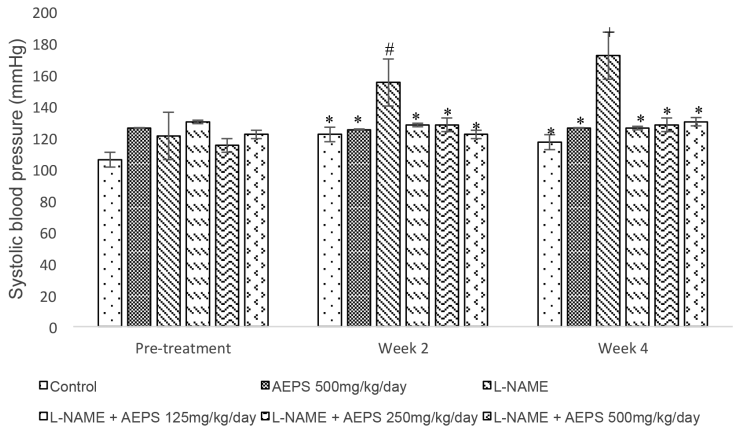
RESULTS

EFFECT OF AEPS ON SYSTOLIC BLOOD PRESSURE

At baseline, there was no significant difference in SBP among the groups. Following two weeks of treatment, L-NAME-induced rats had higher SBP (155.3 ± 2.14 mmHg) compared to control rats (121.8 ± 0.60 mmHg) ($p < 0.001$). Treatment of L-NAME-induced rats with all three doses of AEPS (125, 250 and 500 mg/kg/day) successfully reduced SBP to normal level (128.0 ± 4.37, 127.8 ± 5.19 and 122.17 ± 4.48 mmHg, respectively) ($p < 0.001$). At the end of four weeks treatment, the SBP of L-NAME-induced rats were persistently high compared to control (172.3 ± 5.06 vs. 116.7 ± 1.52 mmHg, $p < 0.001$). Treatment with all three doses of AEPS significantly attenuated L-NAME-induced rise in SBP at the end of treatment (126.0 ± 5.2, 127.83 ± 3.79 and 129.67 ± 3.74 mmHg, respectively) ($p < 0.001$). However, there was no significant difference in SBP between the three doses of AEPS following two and four weeks of treatment. In addition, administration of AEPS alone did not significantly alter the SBP compared to control.

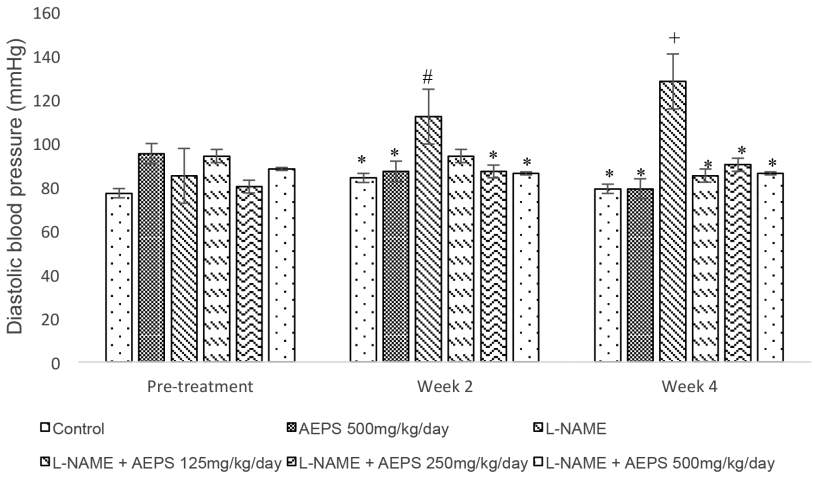
EFFECT OF AEPS ON DIASTOLIC BLOOD PRESSURE

At baseline, there was no significant difference in DBP among the groups. Following two weeks of treatment, L-NAME-induced rats had higher DBP (112.2 ± 3.25 mmHg) compared to control rats (83.6 ± 1.23 mmHg) ($p < 0.01$). Treatment of L-NAME-induced rats with three doses of AEPS (125, 250 and 500 mg/kg/day) successfully reduced DBP (94.0 ± 5.41, 87.0 ± 4.97 and 86.3 ± 8.2 mmHg) ($p < 0.05$). At the end of the four weeks treatment, the DBP of L-NAME-induced rats were persistently high compared to control (127.5 ± 3.93 vs. 78.8 ± 2.41 mmHg, $p < 0.01$). Treatment of L-NAME-induced rats with all three doses of AEPS significantly reduced DBP to normal level at the end of treatment (84.5 ± 4.38, 90.0 ± 2.44, and 86.3 ± 4.19 mmHg, respectively) ($p < 0.05$). However, there was



*$p < 0.001$ versus L-NAME group within the same week, # $p < 0.001$ versus pre-treatment group

FIGURE 1. Effect of AEPS on systolic blood pressure (SBP) in L-NAME induced hypertensive rats



*p<0.05 versus L-NAME within the same week, # p<0.01 versus pre-treatment group

FIGURE 2. Effect of AEPS on diastolic blood pressure (DBP) in L-NAME induced hypertensive rats

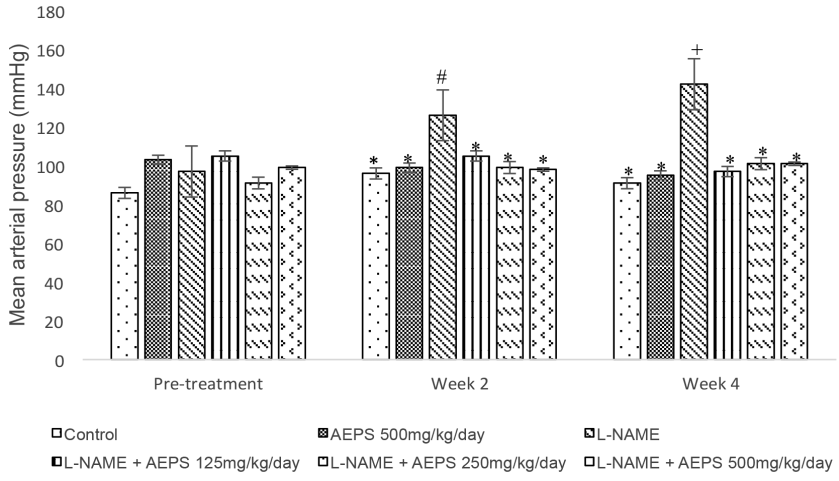
no significant difference in DBP between the three doses of AEPS following two and four weeks of treatment. In addition, administration of AEPS alone did not significantly alter the DBP compared to control.

EFFECT OF AEPS ON MEAN ARTERIAL BLOOD PRESSURE

The changes in MAP were in accordance to changes in SBP and DBP. At baseline, there was no significant difference in MAP among the groups. Following two weeks of treatment, L-NAME-induced rats had higher MAP (126.0 ± 2.45 mmHg) as compared to control rats (96.7 ± 0.88 mmHg) (p<0.05). Treatment of L-NAME-induced rats with all three doses of AEPS successfully reduced MAP (104.7 ± 4.97, 98.8 ± 3.93 and 97.8 ± 6.89 mmHg) (p<0.05). At the end of the four weeks treatment, the MAP of L-NAME-induced rats were persistently high compared to control (142.0 ± 4.49 vs. 91.2 ± 1.78 mmHg, p< 0.05). Treatment of L-NAME-induced rats with all three doses of AEPS significantly reduced MAP at the end of treatment (97.0 ± 3.44, 101.2 ± 1.86 and 100.5 ± 2.71 mmHg, respectively) (p<0.05). However, there was no significant difference in MAP between the three doses of AEPS following two and four weeks of treatment. In addition, administration of AEPS alone did not significantly alter the MAP compared to control.

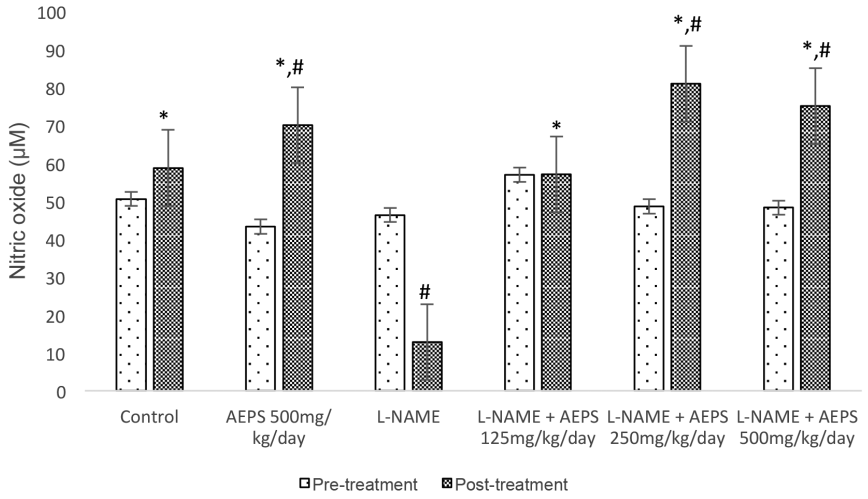
EFFECTS OF AEPS ON SERUM NO

L-NAME induction caused reduction of serum NO compared to its pre-induction level (4.5 ± 1.92 vs 40.7 ± 6.26 μM, p<0.05) as well as compared to control (p<0.001). Treatment with all three doses of AEPS (125, 250, 500 mg/kg/day) increased serum NO compared to L-NAME group (p<0.001) with the values of 56.33 ± 9.15, 80.88 ± 8.55 and 75.02 ± 8.46 μM, respectively. Apart from that, there was significant difference in the level of NO before and after treatment in AEPS, L-NAME + AEPS 250 mg/kg/day and L-NAME + AEPS 500 mg/kg/day groups (p<0.05).



*p<0.05 vs L-NAME within the same week, # p<0.05 vs pre-treatment group

FIGURE 3. Effect of AEPS on mean arterial pressure (MAP) in L-NAME induced hypertensive rats



*p<0.001 versus L-NAME, # p<0.05 versus pre-treatment

FIGURE 4. Effect of AEPS on serum NO in L-NAME- induced-hypertensive rats

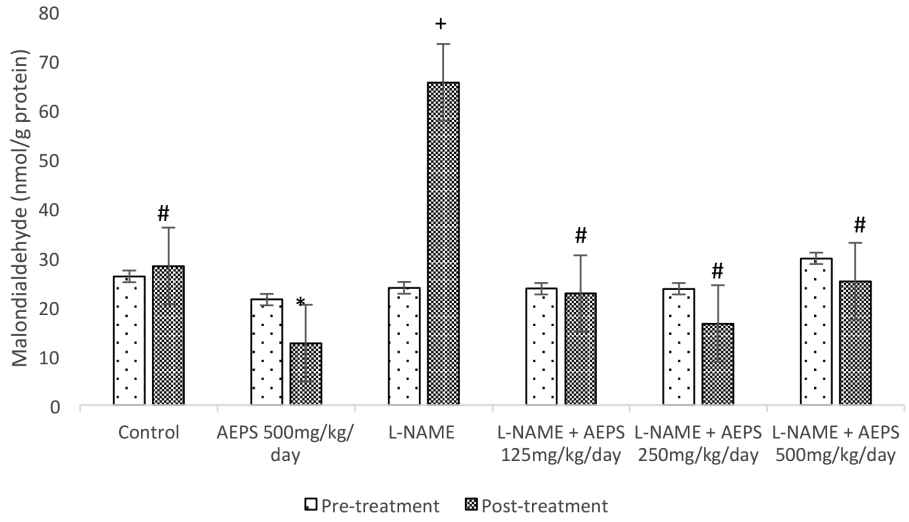
EFFECT OF AEPS ON SERUM MDA

Four weeks following L-NAME induction, serum MDA was increased compared to its pre-induction level (65.59 ± 5.46 vs. 23.81 ± 4.33 nmol/g protein, p<0.01) as well as compared to control (p<0.05). Treatment with all three doses of AEPS (125, 250, 500 mg/kg/day) reduced serum MDA level compared to L-NAME group (p<0.05) with the values of 22.70 ± 3.63, 16.57 ± 4.64 and 25.15 ± 11.39 nmol/g protein, respectively.

DISCUSSION

Hypertension is categorized into primary or essential hypertension (EH) and secondary hypertension. More than 90% of hypertensive patients have EH whereby the cause is still unclear. Current studies suggest that NO deficiency contributes to EH (Arora et al. 2009). Increased in free radical generation inactivates prostacyclin and NO, hence causing half-life of prostacyclin and NO to be decreased. This situation may lead to increase in peripheral vascular resistance and subsequently hypertension (Kumar & Das 1993). Basal production of NO was reduced in spontaneously hypertensive rats (SHR) (Dohi et al. 1990). The endothelium-dependant vasodilator responses were attenuated in patients who suffered from essential hypertension which was mainly contributed to reduced bioactivity of NO (Panza et al. 1990).

The main objective in this study was to evaluate whether the high blood pressure in NO-deficient hypertensive rats induced by L-NAME, an L-arginine analogue that inhibits nitric oxide synthases could be improved by AEPS treatment. In the present study, the administration



*p<0.001 vs L-NAME; # p<0.05 vs L-NAME; + p<0.01 vs pre-treatment

FIGURE 5. Effect of AEPS on serum MDA level in L-NAME induced-hypertensive rats

of L-NAME in drinking water had induced hypertension in rats which concurred with previous studies (Raja 2010). Concomitant treatment with serial doses of AEPS in the L-NAME-induced hypertensive rats had significantly attenuated the hypertension.

Nitric oxide produced by endothelial nitric oxide synthase (eNOS) is the major source of circulating NO. L-NAME interferes with the activity of eNOS, thus reducing NO production (Raja 2010). Since NO is a potent vasodilator, decreased NO production may impair endothelial-dependent vasodilation, causing increased peripheral resistance and blood pressure (Park & Park 2015). Previous study had proven that PS increased NO level by stimulating eNOS expression and activity in HUVECs (Ugusman et al. 2010). Therefore, increased level of NO in L-NAME-induced hypertensive rats treated with AEPS could be due to increase in the activity of eNOS. A study by Ugusman et al. (2014) had found that a flavonoid, rutin, one of the compound in *Piper sarmentosum*, may improve endothelial function by augmenting NO production in HUVEC. Another recent study on PS showed that the extract was able to reduce blood pressure by increasing NO production in SHR (Zainudin et al. 2015).

Apart from inhibiting NO synthesis, L-NAME induces hypertension by causing oxidative stress. L-NAME is responsible to cause imbalance in renin-angiotensin system (RAS) whereby it increased the expression of angiotensin II and also caused renal dysfunction (Rincon et al. 2015). The excessive production of angiotensin II leads to increase vascular superoxide (O_2^-) formation through increased expression of NADPH-dependent oxidase in aortic smooth muscle cells (Tsai et al. 2016). The excessive O_2^- react rapidly with NO to form peroxynitrite ($ONOO^-$). Peroxynitrite is a strong pro-oxidant molecule which causes lipid peroxidation and tissue injury (Hogg et al. 2017). In addition, hypertension itself leads to enhanced production of ROS. Previous studies had shown that in different models of systemic hypertension, there would be enhancement of O_2^- and superoxide-producing enzymes, regardless of how the hypertension was induced (Drummond & Sobey 2014; García-Redondo et al. 2016). Malondialdehyde exists in the serum, plasma, tissues as well as in the urine. It is the commonest analytic estimation of lipid peroxidation and oxidative stress that has been reported (Chen et al. 2015). In the present study, the level of MDA was increased in the L-NAME-induced hypertensive rats; indicating that oxidative stress plays an important role in the pathophysiology of hypertension (Baradaran et al. 2014). Treatment of L-NAME-induced hypertensive rats with AEPS caused reduction in MDA level. This finding is in accordance to a recent study which showed that PS significantly reduced MDA level in spontaneously hypertensive rats (Zainudin et al. 2015).

Decreased level of MDA in AEPS treated group could be attributed to the antioxidant effects of PS. PS had been proven to have strong antioxidant activity (Ugusman et al. 2012). Besides, PS had been shown to suppress intercellular adhesion molecule-1 (ICAM-1) and NADPH oxidase 4 (Nox4) expressions in oxidative stress-induced HUVECs. Nox4 is the predominant enzyme for ROS production in endothelial cells (Ugusman et al. 2010). Several chemical compounds with antioxidant activities found in PS are polyphenols, vitamins C and E, carotenes, tannins, xanthophylls, flavonoids and amides (Hussain et al. 2015). Polyphenols had been shown to reduce blood pressure in NO-deficient model of hypertension (Bernátová et al. 2002; Rodrigo et al. 2016). Since this study used crude extract of PS and not its isolated bioactive components, this study was unable to specify the active compound responsible for the antihypertensive effect. However, it is suggested that the antihypertensive effect of PS in NO-deficient model of hypertension observed in this study is due to its polyphenols content.

Conclusion

AEPS reduces blood pressure in L-NAME-induced hypertensive rats and the antihypertensive effect may be partly mediated by increased NO and reduced oxidative stress. Our findings suggest that AEPS has the potential to be developed as a therapeutic agent for hypertension. However, further studies using isolated bioactive components from AEPS are required in order to support the therapeutic potential of AEPS for hypertension.

Acknowledgements

This study was funded by UKMMC Fundamental Research Fund (FF-174-2011). The authors express their sincere thanks to the staff of Physiology Department; Norizam Salamt, Fadzilah Mohd Suratman, Zanariyah Asmawi, Musmarlina Omar, Aini Farzana Zulkefli and Kamariah Othman for the technical assistance.

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Received: 25 March 2018
Accepted: 6 June 2018