ORIGINAL ARTICLE

Factors Regulating Nitric Oxide Production in Spontaneously Hypertensive Rats Treated with *Piper Sarmentosum* Aqueous Extract

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ABSTRACT

Introduction: Hypertension is a major risk factor for cardiovascular diseases which is one of the leading causes of death worldwide. *Piper sarmentosum* (PS) has been widely used in traditional medicine with proven antihypertensive and antioxidant effects. This study aims to evaluate the antihypertensive potential of PS aqueous extract (PSAE) and to investigate the factors modulating nitric oxide (NO) production through its anti-oxidant activities. **Methods:** PS leaves were extracted with distilled water, freeze-dried and examined to quantify their antioxidant activities through 2,2-diphenyl-1-picrylhydrazyl and ferric reducing ability plasma test. The antihypertensive effect of PSAE in spontaneous hypertensive rats (SHR) was evaluated using four different groups (n=6); C (negative control), K (PSAE 500mg/kg), P (3 mg/kg perindopril) and M (PSAE 500 mg/kg + 1.5 mg/kg perindopril). PSAE and other treatments were given via oral gavage for 28 days. The blood pressure (BP) was determined using the non-invasive BP monitoring tail cuff technique and recorded weekly. SHR's blood was collected to determine the serum NO level using Griess assay. Asymmetric dimethylarginine (ADMA) and arginine levels were determined using high performance liquid chromatography. **Results:** The extract showed good in-vitro antioxidant activities and a significant reduction in both systolic and diastolic BP compared to control group. They were also a decrease in plasma ADMA and an increase in serum NO level. Meanwhile, arginine level does not change significantly. **Conclusion:** High in-vitro antioxidant activities in PSAE enhances the clearance of ADMA that leads to an increase in serum NO production hence ameliorating the blood pressure of SHR.

KEYWORDS: *Piper sarmentosum*, antioxidant, hypertension, nitric oxide, asymmetric dimethylarginine

INTRODUCTION

Systemic arterial hypertension (HPT) is the most prevalent chronic cardiovascular diseases (CVD) in Malaysia and worldwide. World Health Organization (WHO) estimated that globally, almost 7.6 million deaths due to HPT were annually recorded. More than one billion patients around the world, including over 5.8 million Malaysians have high blood pressure (BP).[2,3] Hypertension is an important modifiable risk factor for stroke, myocardial infarction, coronary heart disease and renal failure. For decades, HPT has been extensively investigated in clinical and preclinical studies to understand the mechanisms of the disease as well as to test new potential drugs. Several animal models of HPT have been developed. One of these models is the spontaneously hypertensive rats (SHR), which is widely used as an essential hypertension model to study the antihypertensive potential of natural products.

Herbal products have gained increasing popularity in the last decade and are now used by approximately 20% of the population.[4] In the 21st Century, 11% of the 252 drugs approved by WHO have originated from plants.[5] Many researches on plant extracts have exhibited that natural products contain natural active compounds to treat diseases including HPT. The importance of investigating the mechanism of action of natural products is to provide an idea of how these compounds work in our body.

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Hypertension is a multifactorial disease that includes the interaction between pathophysiological, environmental and genetic susceptibility. There is strong evidence that endothelial dysfunction (ED) plays an important role in the pathogenesis of HPT. Endothelial cells produce nitric oxide (NO) as a response to the increase of BP, which leads to vasodilatation. NO is produced by a healthy endothelial cell as a signalling molecule to dilate blood vessel, ensure optimum blood supply and reduce BP. ED occurs as a result of imbalance between antioxidant availability and free radicals which causes oxidative stress. Reduce NO availability due to oxidative stress is a hallmark of ED.[6]

Several researchers found that many plants and herbs including *Piper sarmentosum* (PS) has high antioxidant activity.[7] They also suggested that additional antioxidant from plants may help in ameliorating blood pressure and able to prevent the development of HPT. There are various methods used to measure the antioxidant activity of a plant including 2,2-diphenyl-1-picrylhydrazyl (DPPH) test, ferric reducing ability plasma (FRAP) test and the screening of total phenolic content (TPC) and total flavonoids content (TFC).

PS is a terrestrial herbal plant. It is called 'kaduk' in Malaysia. It is one of the natural products that shows good prospects to cure diseases including HPT. *Piper sarmentosum* aqueous extract (PSAE) has been shown to increase NO bioavailability with decrease malondialdehyde (MDA) level.[8] Lack of research to investigate the mechanism of PS motivates this research which aims to evaluate the activity of PSAE in reducing blood pressure and examining possible mediated effects of NO production.

MATERIALS AND METHODS

Preparation *Piper Sarmentosum* Aqueous Extract (Psae) of Leaves

PS leaves aqueous extract were prepared at Integrated Centre for Research Animal Care and Use (ICRACU) laboratory, IIUM. PS leaves were cut from its branches and cleaned from extraneous matter with tap water. They were kept at room temperature for three days then placed in a hot-air oven at 40 °C for 3 hours. The dried PS leaves were blended into powder by using an electrical home blender (Philips Hand Blender HR1328/90). A quantity of 100 g of blended powder of PS was added in 900 ml of distilled water to prepare a solution for the extraction process. The solution was concentrated on a magnetic stirrer (Medite, model OTS-40) for 3 hours at 80°C, then the concentrated PS solution was filtered by using a mash filter, then it was stored at –80° C (Haier, DW86L490) for 24 hours. Subsequently, it was kept in the freeze dryer machine (Martin Christ, model Alpha 1-2 LD plud) for seven days. After that the extract was kept on –20° C until use.[9]

Determination of 2,2-Diphenyl-1-Picrylhydrazil (Dpph) Scavenging Activity of Psae

The DPPH activity was measured according to the method of Blois with some modifications.[10] A serial dilution was done on 2 mg of PSAE and 50 µL of sample were added into 96-well microplates. 50 µL of 1mg ascorbic acid dissolved in distilled water was used as positive control while the solvent of the sample extract was used as negative control. 50 µL 0.2 mM of DPPH reagent was added in the dark at room temperature. After 30 minutes, the absorbance value was measured at 517 nm. The activities of the samples were expressed as percentage inhibition of DPPH, were calculated according to this formula. Where A0 and A1 are the absorbance of the control and the test sample values, respectively.

$$\% \text{ Inhibition} = \frac{A0 - A1}{A0} \times 100$$

Determination of Ferric-Reducing Antioxidant Power Activity (Frap) Of Psae

The FRAP assay was done as reported by Thaipong et al., 2006 with some modifications.[11] The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2$ _ $3H_2O$ and 16mL $C_2H_4O_2$), pH 3.6,10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3$_$6H_2O$ solution. The fresh working solution was prepared by mixing 25mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$ _ $6H_2O$ solution and then warmed at 37°C before using. 2 mg PSAE were dissolved in 1 ml of D/W. 140 µl of D/W was pipetted into 96-well microplates and 20 µl of PSAE 2 mg/ml and 40 µl of

FRAP reagent was added. The 96-well microplate was kept in the dark for 20 min. The absorbance value was measured at 593 nm and the standard curve was linear between 0.2 to 12 mM $FeSO_4$. Results are expressed in mM Fe (II)/ g extract and compared with ascorbic acid as standard.

Measurement of Blood Pressure Indices

Blood pressure was measured non-invasively using the tail-cuff technique by CODA non-invasive blood pressure system (Kent Scientific Corporation, USA). The arterial blood pressure was obtained by recording the systolic and diastolic blood pressure readings. Throughout the period of the experiment, animals' temperature was kept stable using warming platform. The temperature was measured every 15 minutes with IR thermometer and maintained at (28 ±1˚C). The measurement of blood pressure was done in the morning before the commencement of treatment and repeated every seven days until the end of the experiment.

Determination of Plasma Asymmetric Dimethylarginine (Adma) by High Performance Liquid Chromatography (Hplc)

HPLC analysis was performed using Agilent 1200 Series (USA) the instrument was equipped with quaternary pump type G1311A combined with a variable wavelength detector type G1314B, an HP 1200 series auto sampler type G1329A, and an Agilent 1200 series vacuum degasser. The column compartment is provided with column thermostat oven type G1316A. The mobile phase consisted of solvent A (Phosphate Buffer 25 mM pH6.5, 8.5% acetonitrile) and solvent B (50% acetonitrile). Chromatographic separation of the analytes was obtained using Agilent C18 (150 × 4.5 mm, 5 µm) column and the temperature was maintained at 30˚ C. The flow rate was 2 mL/min. The injection volume was 20 µl in full loop injection mode. The elution was performed using gradient condition. The analysis time was 22 min and the fluorescence detector was used with excitation wavelength 340 nm; emission wavelength 455 nm.

Total Nitrite and Nitrate Determination

NO level was investigated by using Griess assay by determining total nitrite and nitrate in serum.[12] Briefly, 100 µl of deproteinized sample or serial dilution of standard was added in a 96 well plate, then 100 µl vanadium (III) chloride(8mg/ml) (for conversion of nitrate to nitrite) was added, followed by 50 µL sulfanilamide (2%) and 50 µL N-(1-Naphthyl) ethylendiamine dihydrochloride (0.1%) and incubated at 37˚C for 30 min. Then, the absorbance was read at 540 nm wavelength using the Infinite M200 Nano Quant, multidetection microplate reader. NO level was calculated according to the standard curve of sodium nitrite (0-200 µmol/l).

STATISTICAL ANALYSIS

The data from this study were analysed using Statistical Package for the Social Sciences IBM SPSS software, version 20.0. The mean comparisons of the data were done using multiple measures and one-way analysis of variance (ANOVA) followed by post hoc Tukey HSD test. Data were expressed as mean ± SD. Statistically, the significance level was taken to be p-value less than 0.05.

RESULTS

Antioxidant Activity by Dpph Radical Scavenging Activity and Ferric Reducing Antioxidant Power

The *Piper sarmentosum* aqueous extract (PSAE) DPPH scavenging activity was 69.9 ±0.95% in vitro. Meanwhile the reducing potential of PSAE by the ferric reducing antioxidant power (FRAP) was 97.08 ± 2.82 mM Fe (II)/g.

Systolic Blood Pressure

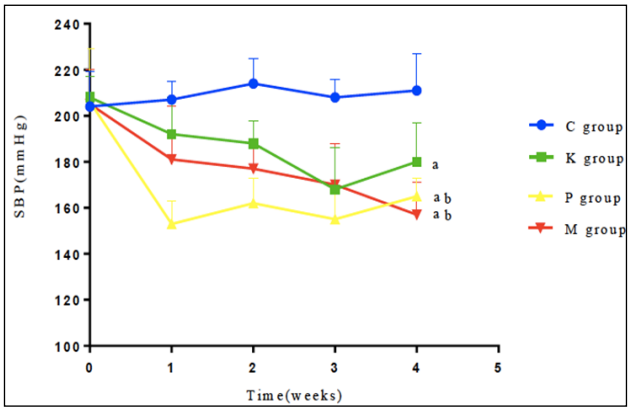
Figure 1(a) shows the systolic blood pressure (SBP) of SHR treated with PSAE. After four weeks of treatment, the SBP of the negative control group (Group C) remain constant. All K, P and M groups showed a significant reduction in SBP compared to the negative control group. However, the SBP of PSAE treated group (Group K) still showed significant difference compared to both positive control (Group P) and mix PSAE and perindopril group (Group M).

Diastolic Blood Pressure

Figure 1(b) shows the diastolic blood pressure (DBP) of SHR treated with PSAE. After four weeks of treatment, the DBP of the negative control group (Group C) remains between (150-160 mmHg). The

DBP of all K, P and M groups were significantly reduced compared to the negative control group. However, the DBP of PSAE treated group (Group K) still showed significant difference compared to both positive control (Group P) and mix PSAE and perindopril group (Group M).

(a)



(b)

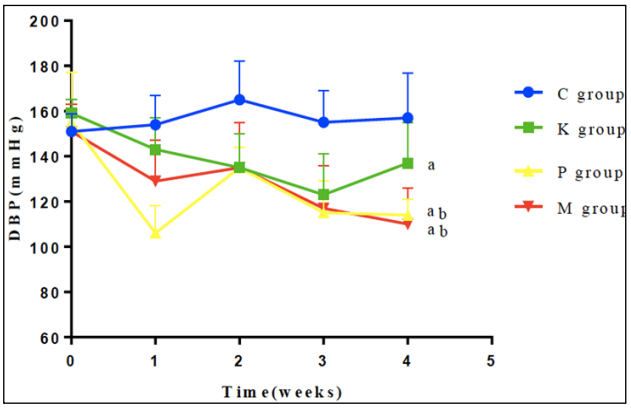


Figure 1. (a) Systolic blood pressure and (b) diastolic blood pressure of SHR treated with PSAE for 28 consecutive days. Note, C group (negative control group); K group (PSAE 500 mg/kg); P group (perindopril 3 mg/kg perindopril); M group (PSAE 500 mg/kg + perindopril 1.5 mg/kg). Data are expressed as mean ± standard deviation (SD). $p<0.05$ was taken as statistically significant at 95% interval. (ANOVA and Tukey test)
[a] significant difference ($p<0.05$) compared to the negative control group (C).
[b] significant difference ($p<0.05$) compared to PSAE group (K).

Serum Nitric Oxide Level

Figure 2 shows the serum NO level of SHR treated with PSAE. The mean of serum NO level was significantly higher in the K, P and M groups compared to the negative control group (Group C). However, there was no significant difference in serum NO level between K, P, and M groups.

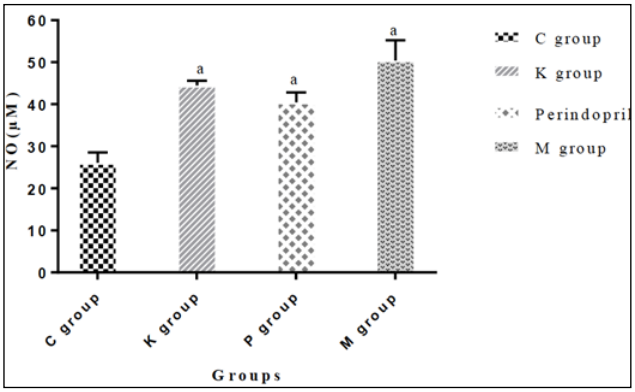


Figure 2. Serum nitric oxide level of SHR treated with PSAE for 28 consecutive days. Note, C group (negative control group); K group (PSAE 500 mg/kg); P group (perindopril 3 mg/kg perindopril); M group (PSAE 500 mg/kg + perindopril 1.5 mg/kg). Data are expressed as mean ± standard deviation (SD). $p<0.05$ was taken as statistically significant at 95% interval. (ANOVA and Tukey test)
[a]significant difference ($p<0.05$) compared to the negative control group (C).

Plasma Asymmetric Dimethylarginine (Adma) Level

Figure 3 shows the plasma ADMA level in SHR treated with PSAE. The negative control group (Group C) showed the highest ADMA plasma level while PSAE (Group K) showed the lowest. All K, P and M groups showed lower ADMA plasma level compared to C group. However, only K group showed a significant difference compared to C group.

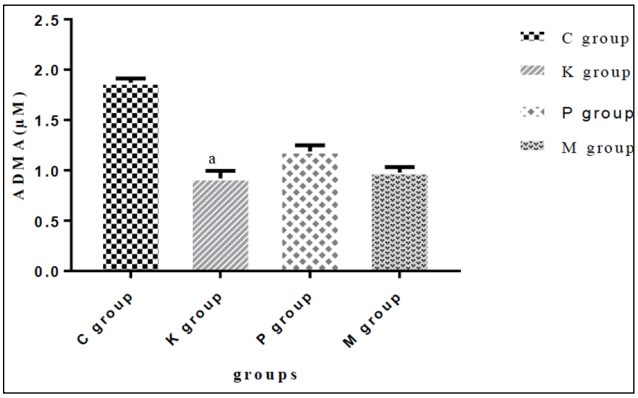


Figure 3. Plasma asymmetric dimethyl arginine (ADMA) level of SHR treated with PSAE for 28 consecutive days. Note, C group (negative control group); K group (PSAE 500 mg/kg); P group (perindopril 3 mg/kg perindopril); M group (PSAE 500 mg/kg + perindopril 1.5 mg/kg). Data are expressed as mean ± standard deviation (SD). $p<0.05$ was taken as statistically significant at 95% interval. (ANOVA and Tukey test)
[a] significant difference ($p<0.05$) compared to the negative control group (C).

Plasma Arginine Level

Figure 4 shows the plasma arginine level in each of the experimental groups. The mean of plasma arginine level of SHR was slightly higher in the control group as compared to other treatment groups (Group K, P and M). However, all treated and control groups showed no significant difference in the level of plasma arginine.

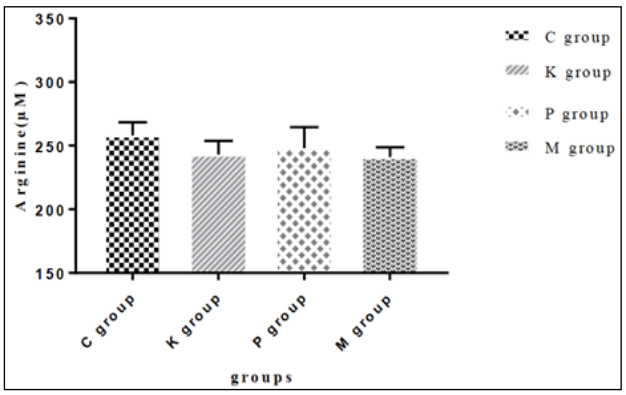


Figure 4. Plasma arginine level of SHR treated with PSAE for 28 consecutive days. Note, C group (negative control group); K group (PSAE 500 mg/kg); P group (perindopril 3 mg/kg perindopril); M group (PSAE 500 mg/kg + perindopril 1.5 mg/kg). Data are expressed as mean ± standard deviation (SD). $p<0.05$ was taken as statistically significant at 95% interval. (ANOVA test)

DISCUSSION

In-Vitro Antioxidant Activity of *Piper Sarmentosum*

Natural product from herbal plants has become more popular nowadays due to their various potentials towards health. Besides that, they cause less side effects and rarely toxicity if taken within therapeutic dose. *Piper sarmentosum* has been proven for its high antioxidant activities and it can be easily found in Malaysia. Meanwhile, the prevalence of HPT in Malaysia is very high as it affects almost half of Malaysian adults aged more than 30 years old. It is a multifactorial disease that causes tremendous morbidity and mortality. One of the main pathogenesis of HPT is the reactive oxygen species (ROS) which causes ED.[13] ROS is an unstable molecule containing oxygen. It freely reacts with any cells, causing cellular injury by attacking cells, enzymes and proteins. This happens when there is an imbalance between ROS and antioxidant availability in the body. This phenomenon causes a number of human cardiovascular disorders such as hypertension, atherosclerosis and diabetic vascular disease.[14]

Normally, the body synthesizes antioxidants, which in turn scavenge these free radicals. However, the antioxidants protect the body when only the degree of free radicals is within the normal physiological range.[7] For this reason, the antioxidant activities of PS are investigated in our study. In general, plants contain different types of free antioxidant compounds. It may include phenolic and flavonoid compounds.[15] PS was found to have high DPPH and FRAP activities in previous studies.[9,16] In our study, PSAE showed good activities in the DPPH and FRAP assays which amount to 69.9%- and 97.08-mM Fe (II)/g respectively. The higher the DPPH, the higher the FRAP contents. Our findings are in congruence with other studies that had reported a high antioxidant activity in PS.[9,15,16]

Antihypertensive Activity of *Piper Sarmentosum* in Spontaneously Hypertensive Rats

We evaluated the antihypertensive effect of PSAE in SHR compared to a therapeutic dose of the angiotensin converting enzyme (ACE) inhibitor (Perindopril) and PSAE + half therapeutic dose of perindopril. We found that PSAE showed a potent antihypertensive activity as it was able to gradually reduce the high BP in SHR although it took a longer duration to achieve the maximum effect at the end of the treatment period. The reduction of DBP was significantly greater than SBP, which implied the improvement of ED as vasodilation effect played crucial roles in decreasing the total peripheral resistance (TPR) in SHR. On the other hand, perindopril 3 mg/kg has shown a potent antihypertensive effect since the 1st week until the end of the treatment period. Similarly, the mix group (PSAE + 1.5 mg/kg perindopril) showed almost the same ability to reverse the elevated BP despite taking 3 weeks to achieve the BP reduction.

These results supported the ability of PS to treat HPT on SHR after the concomitant administration, however, it was still not as potent as perindopril. Nevertheless, PS is also able to reduce asymmetric dimethyl arginine (ADMA) which is not shown by perindopril. This suggests that perindopril has a different mechanism in reducing the BP. Besides that, PSAE also showed that it enhances the synthesis of NO. The dose used in this study was 500 mg/kg once per day by oral administration as it is the optimal dose of antihypertensive activity, according to previous study.[9] It might not be a

therapeutic dose, in which it may be less than the required PSAE dose needed to correct established HPT. In the other hand, may be PSAE treatment should be commenced twice per day to maintain the level of PASE in the blood at a higher level or for a longer duration. Hence, it can be concluded that PSAE administration alone in a small dose act as prophylactic agents against HPT while the combination with a half dose of other established antihypertensive drug showed a synergistic effect that can be used for the treatment of HPT.

Piper Sarmentosum Role Through The Regulation Factors Of Nitric Oxide Production

Healthy endothelial lining is essential to maintain homeostasis of vasodilators and vasoconstrictors. Excessive or unopposed vasoconstrictor effect may lead to increase peripheral resistance, thus increasing the systemic arterial BP. Nitric oxide is a very important and potent vasodilator and it plays a very important role in maintaining a healthy endothelium. NO is synthesized from L-arginine using nitric oxide synthase (NOS) through an enzyme catalytic reaction. ADMA is known for its inhibitory effect on NOS in a variety of cardiovascular diseases and renal dysfunctions which leads to the decrease of NO availability. The dysregulation of the NO and ADMA pathways play an essential role in the change of BP.[17] Significant reduction in serum NO availability leads to the diminished capacity of the endothelial lining function. This causes vasoconstriction hence increasing the resistance of the arterioles which leads to the development of hypertension and cardiovascular diseases.[18]

In this research, PSAE administration increases the NO levels significantly. As such, there is also an increase in the NOS levels.[19] Subsequently, we found a strong relationship between the PSAE treatment and reduction in the ADMA concentration in PSAE and mixed groups compared to the control group. These findings could be due to the effective elimination of ADMA from the body. ADMA is mainly removed from the body by enzymatic degradation through dimethylarginine dimethylaminohydrolase (DDAH), which is present in the kidneys.[20,21] An explanation for such a decrease of ADMA could be due to it being increasingly converted to L-citrulline by the DDAH enzyme through a reduction in the ROS formation.[13]

CONCLUSION

PSAE has high antioxidant activities. These compounds increased the availability of antioxidants, thus reducing the formation of ROS in SHR. Subsequently, this condition enhanced the clearance of ADMA from the body. Low ADMA level decreased the inhibition and competition with eNOS resulting in increased eNOS activated conversion of arginine to NO. This leads to an increase in the production of NO in SHR. High NO bioavailability causes an optimum vasodilation of arterioles, which leads to a reduction of TPR thus ameliorating the BP.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

ACKNOWLEDGMENTS

A special thanks to the technicians of the Laboratory of the Basic Medical Sciences Department, Faculty of Medicine and Assistant Professor Abdul Bashar Helaludin. This study was funded by FRGS/1/2016/WAB11/UIAM/03/1.

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