

Kertas Asli/Original Articles

Syzygium polyanthum Protects Against Hypertensive Induced Kidney Damage in Spontaneous Hypertensive Rat Model

(Perlindungan *Syzygium Polyanthum* terhadap Penyakit Ginjal Kronik Aruhan Hipertensi dalam Model Tikus Berhipertensi Spontan)

LIZA NOORDIN, NURUL SYAHIDA RAMLI, NOR HIDAYAH ABU BAKAR & WAN AMIR NIZAM WAN AHMAD

ABSTRACT

Syzygium polyanthum is traditionally used as anti-hypertensive agent. However, the nephroprotective effects of *S. polyanthum* against hypertensive induced chronic kidney disease has yet to be elucidated. This study was conducted to determine the antioxidant properties and nephroprotective effects of aqueous extract of *S. polyanthum* (AESP) in the spontaneous hypertensive rat model (SHR). The phytochemical constituent was identified using the phytochemical screening and HPLC methods. The *in vitro* antioxidant activities were determined by DPPH radical scavenging and ferric reducing antioxidant power (FRAP) assays. Fifty male SHR were equally divided into 5 groups, ($n=10/\text{group}$); Untreated-SHR, 20 mg/kg Losartan-treated SHR, 1500 mg/kg AESP treated SHR, 1750 mg/kg AESP treated SHR and 2250 mg/kg AESP treated SHR, while 10 male Wistar Kyoto rats (WKY) were used as control. Losartan and AESP were administered by oral gavage. Rats were sacrificed after 12 weeks of experiment. The phytochemicals include phenolics, flavonoids and alkaloids were identified. AESP has high antioxidant activity as shown by antioxidant assays. AESP normalised systolic blood pressure ($p<0.05$) and significantly improved renal function ($p<0.05$). AESP also significantly reduced malondialdehyde (MDA) ($p<0.05$) and increased superoxide dismutase (SOD) levels in the serum as compared to untreated-SHR group ($p<0.05$). Ultrastructure of renal damage improved by supplementation of AESP. Conclusively, *S. polyanthum* is potential to alleviate hypertensive induced chronic kidney disease through its antioxidant properties.

Keywords: Antioxidant; nephroprotective; spontaneous hypertensive rat; *Syzygium polyanthum*

ABSTRAK

Syzygium polyanthum digunakan secara tradisi sebagai agen anti-hipertensi. Walau bagaimanapun, kesan perlindungan pada ginjal dalam penyakit ginjal kronik aruhan hipertensi masih perlu dilakukan. Kajian ini dijalankan untuk mengenalpasti pelbagai ciri antioksidan dan kesan perlindungan pada ginjal oleh ekstrak akues *S. polyanthum* (AESP) dalam model tikus berhipertensi spontan (SHR). Komponen fitokimia dikenalpasti menggunakan saringan fitokimia dan teknik HPLC. Aktiviti *in vitro* antioksidan dikenalpasti melalui asai penangkapan radikal bebas DPPH dan asai kuasa antioksidan penurunan ferik (FRAP). Lima puluh ekor tikus jantan SHR dibahagikan sama rata kepada 5 kumpulan, ($n=10/\text{kumpulan}$); SHR tidak dirawat, SHR dirawat dengan 20 mg/kg Losartan, SHR dirawat dengan 1500 mg/kg AESP, SHR dirawat dengan 1750 mg/kg AESP dan SHR dirawat dengan 2250 mg/kg AESP, manakala 10 ekor tikus jantan Wistar Kyoto rats (WKY) telah digunakan sebagai kumpulan kawalan. Semua tikus dikorbankan selepas 12 minggu tempoh eksperimen. Kandungan fitokimia termasuk fenolik, flavonoid dan alkaloid telah dikenal pasti. AESP mempunyai aktiviti antioksidan yang tinggi ditunjukkan melalui asai antioksidan. AESP menormalkan tekanan darah sistolik dan menambah baik fungsi ginjal dengan signifikan. AESP juga menurunkan aras malondialdehid (MDA) dan meningkatkan aras superoksida dismutase (SOD) dalam serum dengan signifikan berbanding kumpulan SHR yang tidak dirawat. Histologi ginjal yang rosak dipulihkan dengan suplemen AESP. Kesimpulannya, *S. polyanthum* boleh digunakan untuk mengurangkan penyakit ginjal kronik aruhan hipertensi melalui pelbagai ciri antioksidan.

Kata kunci: Antioksidan; perlindungan pada ginjal; *Syzygium polyanthum*; tikus berhipertensi spontan

INTRODUCTION

Hypertension, a chronic increase in blood pressure above 140/90 mmHg, remains a major health challenge and

burden worldwide (Whelton et al. 2017; Mahadir Naidu et al. 2019). This disease is one of the major public health problems with global prevalence which increased from 594 million to 1.13 billion people in 1975 and 2015 respectively

(NCD Risk Factor Collaboration 2017). These numbers are expected to remain high by 2025, with approximately 1.56 billion people will have hypertension (Tabrizi et al. 2016). Despite government policies and hypertension task forces, the prevalence of hypertension in Malaysia has been persistently high (Mahadir Naidu et al. 2019). Hypertension also contributes to various complications such as stroke, myocardial infarction, heart failure, peripheral arterial disease and end-stage renal disease, which increase the risk of death if not treated early (Adler et al. 2015). Arterial hypertension is associated with end-organ damaged, including vasculopathy, cerebrovascular damage, heart disease and nephropathy (Schmieder 2010).

Evidence has accumulated that the prevalence of a bi-directional relationship between hypertension and related organ damage is increasing worldwide. Hypertension is recognized as a major cause and effect of kidney disease (Pugh et al. 2019). Systemic hypertension is known to cause glomerular hypertension that manifests as high perfusion pressure, high filtration pressure, and high transmembrane pressure in glomeruli (Endlich & Endlich 2012). Typically, blood pressure (BP) rises with decreased kidney function and sustained elevation in BP causes progression of kidney disease (Judd & Calhoun 2015). The impact of uncontrolled hypertension is mainly associated with several renal morphological changes, particularly abnormalities in glomerular, renal vessel, renal tubules and interstitial tissues disorders (Mullins et al. 2016). Multiple factors have been attributed to the pathophysiology of chronic kidney disease (CKD) associated hypertension, including sodium dysregulation, increased sympathetic nervous system and alterations in renin-angiotensin aldosterone system activity (Hamrahian & Falkner 2016). Hypertensive nephrosclerosis is a common leading cause of end-stage renal disease (Hart & Bakris 2010; Murea & Freedman 2010).

It is important to control the progression of hypertension in patients with renal disease as it slows the disease progression (Pugh et al. 2019). The need for antihypertensive drugs is not limited only to control blood pressure, but also to prevent complications of hypertension (Weisfeldt & Zieman 2007). According to the World Health Organization, around 80% of the world's population relies on herbal products as primary health care (Ekor 2014). It has been reported that up to 70,000 plant species have been screened for biological activity and about 70% have been commercialized (Nordin et al. 2019). The ethno-medicinal study of plants continues to expand rapidly throughout the world, bringing many new products into the market. *Syzygium polyanthum* (Wight) Walp. var. *polyanthum* or commonly referred to as "serai kayu" that belongs to the family of Myrtaceae, is one of the medicinal plants that has been linked to the treatment of hypertension. The

plant is commonly distributed in tropical regions of Asian countries, including Malaysia (Ismail & Wan Ahmad 2019), Indonesia (Widyawati et al. 2015), Thailand (Suksri et al. 2005) and Singapore (Wong et al. 2006).

The leaves are well known for the treatment of various diseases such as diarrhea, hypertension, gastritis, hypercholesterolemia and diabetes mellitus (Sumono & Agustin 2008; Khan et al. 2009; Ismail & Wan Ahmad 2019). Extracts of the leaves have been reported to exhibit anti-hypertensive effect (Ismail et al. 2013), antifungal activity (Kusuma et al. 2019), antibacterial activity (Grosvenor et al. 1995) and antitumor activity (Ali et al. 2000). However, the hydromethanolic extract of this plant has been shown to have weak antibacterial and cytotoxic activities on 4T1 and MCF-7 mammary carcinoma cells (Nordin et al. 2019). Studies of antioxidant activities of *S. polyanthum* have also been reported (Wong et al. 2006; Kusuma et al. 2011; Safriani et al. 2015). However, no studies have been reported on possible nephroprotective effects in hypertensive induced chronic kidney disease. The aim of this study was therefore to determine the nephroprotective effects of aqueous extracts of *S. polyanthum* leaves (AESP) in spontaneously hypertensive (SHR) rats.

MATERIALS AND METHODS

MATERIALS

DPPH was purchased from Friendemann Schmidt, Australia, Gallic acid from Sigma-Aldrich, China, Quercetin and Glacial acetic acid from Sigma-Aldrich, Germany, 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) from Sigma-Aldrich, Switzerland, Folin-Ciocalteu (F-C) reagent from Sigma-Aldrich, USA, Losartan from Merck Sharp & Dohme Ltd., United Kingdom. McDowell Trump fixative from Merck, USA. All other chemicals were of the highest analytical grade available from local suppliers.

PREPARATION OF AQUEOUS EXTRACT OF *SYZYGIUM POLYANTHUM*

The leaves of *S. polyanthum* were collected at District of Kota Bharu, Kelantan, Malaysia. The plant was identified and authenticated by a botanist, Dr. Richard Chung from Forest Research Institute Malaysia (FRIM). The voucher specimen of dried leaves was then deposited in FRIM herbarium with the identification number: PID-171011-10. The cleaned leaves were dried in an incubator at 50°C for three consecutive days until constant weight was achieved. The dried leaves were ground into powder using laboratory

grinder. An aqueous extract of *S. polyanthum* (AESP) was prepared by using ultrasound-assisted extraction technique. There were three cycles involved in the process of extraction. Sample of 200 g of the plant powder was boiled three times with 400 mL, 250 ml and 150 ml of distilled water at 80°C. For each cycle, the mixture was boiled for 30 minutes, stirred thoroughly, sonicated for 30 minutes and filtered via Whatman paper No. 1. The resultant filtered extract was frozen overnight at -20°C before lyophilized in a freeze-dryer. The yields obtained as AESP were kept in the -20°C refrigerator for phytochemical analysis and antioxidant activity.

PHYTOCHEMICAL ANALYSIS

Phytochemical analysis of AESP was performed using colorimetric method to screen the presence of phenolics, flavonoids, alkaloids, terpenoids, tannins, saponins, steroids, glycosides, cardiac glycosides, quinones, resins, anthraquinone, xanthoproteins and coumarins. A sample of AESP was dissolved in distilled water with a minimum amount of solvent adequate for a solution. The identification of phytochemical groups was performed using previous standardized methods to evaluate the presence for phenolics, flavonoids, tannins, steroids (Doss 2009), alkaloids, anthraquinone, coumarins, saponins, quinones and glycosides (Gul et al. 2017), and terpenoids, cardiac glycosides, resins and xanthoproteins (Ganesh & Vennila 2011).

TOTAL PHENOLIC AND FLAVONOID CONTENTS

Total phenolic and flavonoid contents were done to assess the total content of both compounds in the extracts. The total phenolic contents in AESP was estimated by using Folin-Ciocalteu colorimetric method (Kaur et al. 2008). In this study, gallic acid was chosen as the reference standard of total phenols compound for construction of the calibration curve. Gallic acid stock solution (1 mg/mL) was prepared by dissolving 5 mg gallic acid in 5 mL distilled water. Then, six different concentrations of standard gallic acid were subsequently prepared using distilled water (50–300 µg/mL). An amount of 200 µL of each diluted standard gallic acid and 200 µL of 1 mg/mL extract were added separately into the test tubes followed by addition of 800 µL distilled water. Then, 500 µL of 10% (w/v) Folin–Ciocalteu reagent was added into each tube. After 5 minutes, the solution was mixed with 1.5 mL of 20% w/v sodium carbonate and incubated at room temperature for 2 hours in the dark. The absorbance was measured at 765 nm against the reagent blank using spectrophotometer.

The determinations were conducted in triplicates to obtain a consistent result. The total phenolic content of the AESP was calculated as mg gallic acid equivalents (mg GAE/ 1 g of the dry extract).

The total flavonoid contents in AESP was measured by using aluminium colorimetric method (Kamtekar et al. 2014). Quercetin that has high constituents of flavonoid compounds, was chosen as the reference standard for construction of the calibration curve. Quercetin stock solution (1 mg/mL) was prepared by dissolving 5 mg quercetin in 5 mL methanol. Then, six different concentrations of standard quercetin (50–300 µg/mL) were prepared. 1 mL of each diluted standard quercetin and 1 mL of 1 mg/mL extract were separately pipetted into the test tubes followed by addition of 4 mL distilled water. Subsequently, 0.3 mL of 10% (w/v) sodium nitrate solution was added into each tube and allowed to stand for 5 minutes. Then, 0.3 mL of 10 % (w/v) aluminium chloride solution was added and the reaction mixtures were left for 1 minute. Later, 2 mL of 1 M sodium hydroxide was added and transferred into the cuvette and the absorbance was measured at the wavelength of 420 nm using spectrophotometer. All determinations were done in triplicates. The total flavonoid contents of the aqueous extract (AESP) was calculated as mg quercetin equivalents (mg QE/1g of dry extract).

IN VITRO ANTIOXIDANT ACTIVITY

The *in vitro* antioxidant activities of the extract were assessed by using ferric reducing assay and free radical scavenging in the present study. Ferric reducing antioxidant power (FRAP) Assay: The FRAP response was determined by using previous method (Rajurkar & Hande 2011). The principle is based on the reduction of ferric tripyridyl triazine (Fe³⁺ TPTZ) complex to a blue-colored ferrous (Fe²⁺). The ferrous sulphate stock solution (5 mM) was firstly diluted into six different standard dilutions (0.5–3 mM) using distilled water accordingly. An amount of 50 µL of each diluted standard ferrous sulphate, 50 µL of 1 mg/mL extract and 50 µL of 1 mg/mL of ascorbic acid (positive control) were separately added into the test tubes. Then, 150 µL distilled water was added followed by 1.5 mL FRAP reagent. The FRAP reagent was prepared freshly by mixing 300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl₃.6H₂O in the volume ratio 10:1:1. The mixtures were mixed well and immediately incubated in the ambient room for 30 minutes. The absorbance of the mixtures was measured at the wavelength of 593 nm using spectrophotometer against the reagent blank. All determinations were conducted in triplicates. A calibration curve was then generated by plotting the mean absorbance

versus the standard ferrous sulphate (0.5-3.0 mM). The reducing ability of AESP was expressed as mM ferrous equivalents (mM FE / 1 mg of the dry extract).

DPPH Radical Scavenging Assay: In this study, gallic acid was chosen as the reference standard for the construction of calibration curve and the method was performed based on the previous study (Sharma & Bhat 2009). Both standard gallic acid and AESP stock solution (1 mg/mL each) were prepared. Briefly, 2 mL of each diluted standard gallic acid and 2 mL of each diluted extract were separately added into the test tubes. An amount of 2 mL 0.4 mM DPPH which was diluted in methanol was added into each tube mixed thoroughly and allowed to stand for 30 minutes. The absorbance of the mixtures was measured at the wavelength of 517 nm using spectrophotometer against reagent blank. As a remark, 2 mL of distilled water and 2 mL of methanol was served as the blank solution. Three independent tests were performed, and the mean absorbance was recorded. The percentage of DPPH scavenging activity for both samples were determined.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Quantification of the gallic acid was done using high performance liquid chromatography (HPLC) technique by using Waters Delta 600 with 600 Controller linked to a Waters 996 photodiode array detector (Milford, MA, USA). For chromatographic analysis, a reversed-phase C18 column with length of 4.6 mm i.d x 250 mm was used. Briefly, 10 mg of AESP powder was dissolved in 1 mL of methanol and filtered using 0.45 µm of syringe filter. The mobile phase was a gradient prepared from 0.1% (v/v) aqueous formic acid in HPLC-grade water (component A) and acetonitrile (component B). A gradient was used as 0-12 min, 15% B; 12-22 min, 25% B; 22-25 min, 25% B and 25-35 min, 15% B. Throughout the analysis period, the flow rate was maintained at 1.0 mL/min while the injection volume was maintained at 10 µL. At the same time, gallic acid with concentration of 100 µg/mL was run as a standard.

The analyses were recorded at specific wavelength (280 nm) using photodiode array detector with three replicates. Six serial concentrations of gallic acid (40, 60, 80, 100, 120, and 140 µg/mL) were run. A calibration curve was then generated by plotting the mean absorbance versus the standard gallic acid concentrations. The concentrations of gallic acid in AESP were then calculated and estimated based on the peak areas of AESP in the chromatograms and the linear equation obtained from the standard curve.

ANIMALS

Fifty male spontaneously hypertensive rats (SHR) and 10 male Wistar Kyoto rats (WKY) age 8 weeks (200-250 g) were obtained from Animal and Research Centre (ARASC), Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia. Animals were kept individually in a standard polypropylene cage with a constant temperature at 23 ± 1 °C with 12-hour light-dark cycle. The procedures and protocols were approved by Animal Ethic Committee of Universiti Sains Malaysia (USM/ Animal Ethics Approval/ 2016 (102) (757)) in accordance with the institutional guidelines. Adequate measures were taken to minimize pain or discomfort to the rats. Rats were acclimatized for 7 days and were administered a standard Altromin pellet imported from Germany by Sterling Ascent, Malaysia with water *ad libitum*.

EXPERIMENTAL DESIGN

Ten WKY rats were used as normal control group (WKY). Fifty SHR rats were randomly divided into five groups (n = 10/group); Untreated SHR (received distilled water), Losartan treated (received Losartan at 20 mg/kg), AESP 1500 mg/kg (received AESP at 1500 mg/kg), AESP 1750 mg/kg (received AESP 1750 mg/kg) and AESP 2250 mg/kg (received AESP at 2250 mg/kg). Hypertension starts to develop in SHR at 5–6 weeks of age and blood pressure plateaus at approximately 50–60 days (Dickhout & Lee 1997), thus we have chosen rats at 8 weeks of age in this study. Untreated SHR represents a model of chronic kidney disease secondary to hypertension. SHR is a good model for studying human essential hypertension whereby proteinuria begins to increase at 6 weeks of age in male SHR and increases linearly up to 70 weeks of age (Yang et al. 2010). In addition, this model demonstrated the presence of glomerulosclerosis and interstitial fibrosis starting between 40 and 50 weeks with the GFR decreases by 20% to 30% at 14 to 15 and 30 to 32 weeks of age, respectively. All rats received normal rat chow pellets and water *ad libitum*. Distilled water, Losartan and AESP were administered via oral gavage for 12 weeks. Systolic blood pressure (SBP) was measured bi-weekly using a tail-cuff method (LabQuip, USA) for 12 weeks. Animals were euthanized with 100 mg/kg sodium pentobarbitone via intraperitoneal injection. Blood was collected via cardiac puncture for the measurement of biochemical parameters, full blood count (FBC) and oxidative stress parameters. Kidney was isolated, cleaned from adherent fats and connective tissues for histological study using scanning electron microscope (SEM).

MEASUREMENT OF BIOCHEMICAL PARAMETERS

Blood samples for the measurement of biochemical parameters (Blood urea nitrogen (BUN) and serum electrolytes) and full blood count (FBC) were sent to B.P. Clinical Lab Sdn. Bhd., Kota Bharu, Kelantan. We were provided a plain tube for the measurement of BUN and serum electrolytes, and a tube contained anticoagulant EDTA for measurement of FBC to stop its natural clotting. A volume of 3 mL of whole blood was added into each tube. FBC was measured in whole blood.

MEASUREMENT OF OXIDATIVE STRESS MARKERS

Oxidative stress markers: malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD) and catalase (CAT) were measured in serum. MDA was measured by commercially available kits from Northwest Life Sciences Specialties, Washington, USA. TAC, SOD and CAT were determined by commercially available kits from Bioassay Systems, California, USA.

ULTRASTRUCTURE OF KIDNEY

The ultrastructure of kidney was observed using scanning electron microscope (SEM). One mm³ segment of kidney was used in this experiment, where it is fixed in McDowell-Trump at 4°C for 4- 24 hours (Ramli et al. 2017). Sample was washed with 0.1 M phosphate buffer for 3 times with 10 minutes for each change. Then, it was post-fixed and incubated at 4°C in 1% osmium tetroxide for 2 hours. Sample was then washed with 0.1 M phosphate buffer for 3 times with 10 minutes for each change. Sample underwent dehydration process in a series of ascending acetone concentration (35%, 50%, 75%, 95% and 100%) for 10 minutes each except 15 minutes for 100% acetone. Then, it was leaved to air dried in critical point dryer instrument (Quantum Technologies, E3000). The dried specimen was then mounted onto nickel stubs, sputter-coated with gold and platinum, and observed under SEM (FEI Quanta 450 Scanning Electron Microscope, Thermo Fisher Scientific, USA).

STATISTICAL ANALYSIS

GraphPad Prism (version 8.0 for Windows, GraphPad Software, La Jolla California USA) was used to analyze the data. The normality test was performed to determine whether the data distribution was normally distributed. One-way analysis of variance (ANOVA) test was used

and followed by Tukey's post-hoc test for multiple comparisons. SBP was analyzed using repeated-measures analysis of variance (ANOVA). Values are expressed as means (with standard deviations). All tests were two-tailed and the level of significance was set at $p < 0.05$.

RESULTS

PHYTOCHEMICAL ANALYSES

The phytochemical analyses of AESP revealed the presence of multiple compounds as shown in Table 1. The findings indicate the presence of bioactive components in the AESP.

ANTIOXIDANT ACTIVITY

AESP showed high total phenolic and flavonoid contents as well as high antioxidant activities from ferric reducing antioxidant power (FRAP) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays when compared with the standard reference as shown in Table 2. The total phenolic content in AESP was 232.806 (0.3867) mg gallic acid equivalent (GAE)/gram of dry extract, which suggests AESP has high phenolic contents (Perumal et al. 2012). The total flavonoid content in AESP was 100.260 (1.3354) mg quercetin (QE)/gram of dry extract, which suggests AESP is a good source of flavonoid contents (Perumal et al. 2012). The ferric ion-reducing activity of AESP was 5.274 (0.16) mM FE/ mg of dry extract while the ascorbic acid was 6.675 (0.224). The activity of AESP was comparable to ascorbic acid. AESP also shows a strong DPPH scavenging activity of (93.57 (0.203) %) which was comparable to gallic acid (93.87 (1.44) %).

GALLIC ACID QUANTIFICATION

Figure 1 shows high performance liquid chromatography (HPLC) chromatogram of AESP at wavelength of 280 nm. Multiple peaks (compounds) were observed which were eluted at different time, $t = 2.801, 3.899, 4.778, 8.731, 10.402$ and 15.962 minutes, respectively. The highest peak was observed at $t = 3.899$ minutes, that is similar elution time with typical chromatogram of standard gallic acid ($t = 3.899$ minutes). Hence, the results strongly suggest gallic acid might present in AESP with the highest detection.

SYSTOLIC BLOOD PRESSURE

Figure 2 shows the changes of bi-weekly systolic blood pressure (SBP) for 12 weeks in all groups. The mean

TABLE 1. Phytochemical analyses in *S. polyanthum* leaves extracts

| Phytochemical Constituents | Results |
|----------------------------|---------|
| Phenolics | + |
| Flavonoids | + |
| Alkaloids | + |
| Terpenoids | + |
| Tannins | + |
| Saponins | + |
| Steroids | + |
| Glycosides | + |
| Cardiac Glycosides | + |
| Quinones | + |
| Resins | + |
| Anthraquinones | - |
| Xanthoproteins | - |
| Coumarins | - |

Note: AESP: Aqueous extract of *S. polyanthum* leaves, “+”: Presence of class constituent and “-”: Absence of class constituent

TABLE 2. The total phenolic, total flavonoid and antioxidant activity of AESP

| | Total Phenolic Content (mg GAE g ⁻¹) | Total Flavonoid Content (mg QE g ⁻¹) | FRAP Assay mM FE/ mg) | DPPH Assay (% inhibition) |
|-----------|---|---|--------------------------------|------------------------------|
| AESP | 232.806 (0.3867) | 100.260 (1.3354) | 5.274 (0.16) | 93.57 (0.203) |
| Reference | Gallic acid | Quercetin | Ascorbic acid 6.675 (0.224) | Gallic acid 93.87 (1.44) |

Note: AESP, Aqueous extract of *S. polyanthum* leaves; DPPH, 2,2-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power. The measurement was performed in triplicate. All values are shown in mean (standard deviation)

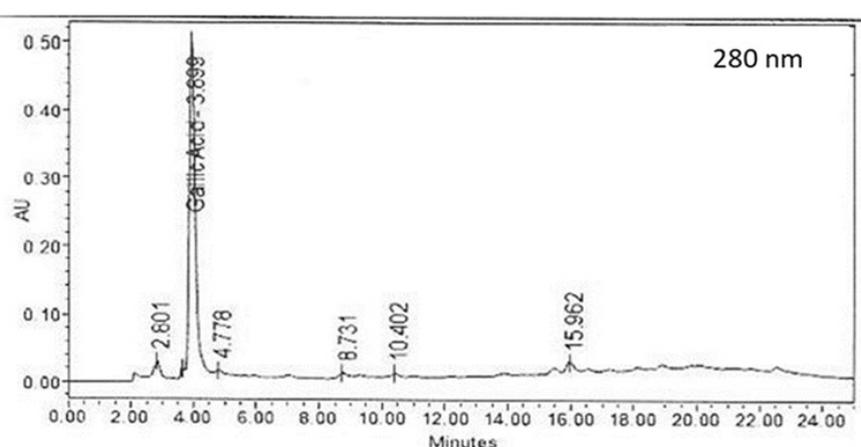


FIGURE 1. HPLC chromatogram of AESP at wavelength of 280 nm with standard reference gallic acid.

SBP of untreated SHR was significantly higher when compared to WKY throughout the study period. There was a significant main effect of time ($F_{4,622}, 249.6 = 915.2; p < 0.0001$), signify a differences in the SBP among the different time points from 2 weeks to 12 weeks. There was also a significant main effect of treatment ($F_{30, 324}$

$= 123.0; p < 0.0001$) whereby SHR treated rats displayed significantly decreased SBP compared to untreated SHR. In addition, SBP from AESP 1750 mg/kg group and AESP 2250 mg/kg group were normalized at week 10, and by week 12, SBP from all AESP treated groups were normalized.

THE EFFECTS OF AESP ON RENAL PARAMETERS

Blood urea nitrogen (BUN), creatinine, uric acid and potassium were significantly higher in untreated SHR when compared to WKY group (Table 3). Losartan and AESP supplementation at all doses significantly reduced the levels of BUN, creatinine and uric acid. However, only treatment with AESP at 1750 mg/kg and 2250 mg/kg significantly reduced the potassium level compared to untreated SHR. There were no significant differences in levels of sodium between the groups.

THE EFFECTS OF AESP ON OXIDATIVE STRESS MARKERS

Table 4 shows the results of oxidative stress markers in the serum. MDA levels were significantly higher in the untreated SHR compared to the WKY group. All the treatment groups showed significant lower of MDA levels compared to untreated SHR. SOD activities were significantly lower in untreated SHR compared to the WKY group. Treatment with Losartan and AESP (1500 mg/kg and 1750 mg/kg) showed significant increased SOD activities compared to untreated SHR. Meanwhile, there was no significant difference in SOD levels between AESP at 2250 mg/kg and untreated SHR. There were no significant differences in the levels of TAC and CAT between the groups.

THE EFFECTS OF AESP ON FULL BLOOD COUNT

There was no significant difference in each parameter between the groups (Table 5).

ULTRASTRUCTURE OF KIDNEY

The ultrastructure of kidney was examined using scanning electron microscope (SEM). Figure 3 demonstrates the images of rat's kidney from all groups. The kidney was viewed under magnification of 2500x and 10,000x. In WKY group, at 2500x magnification (a), 3D image of glomerulus was viewed. The glomerulus was situated within the capsule and well-surrounded by glomerular membrane. It was illustrated as tight ball capillaries surrounded by podocytes. The podocytes exhibit as normal foot processes, which arranged in parallel, longer in size and show numerous foot-like radiating surrounds the capillary loop (known as pedicels). There were (b) no distortion or fusion of podocytes was identified in the 10,000X magnification.

In contrary, in untreated SHR group, the glomerulus appearance was slightly distorted and shrunk (c). Whereas the podocytes appeared large in size and blunted. The foot process was also irregular in thickness and some started to detach from the capillary loop. The pedicels displayed as short, tortuous and not in parallel arrangement (d). On the other hand, in the kidney of the Losartan-treated rat, 1500 mg/kg, 1750 mg/kg and 2250 mg/kg AESP-treated rat, there were marked improvement of the glomerular appearance and size. The Bowman's space looked more defined and the podocytes showed regular in arrangement (e), (g), (i), (k). All treatment groups also showed normal appearance of podocytes; illustrating as parallel, longer and thin in size (f), (j), (l) which similar like podocytes structure of WKY except for 1500 mg/kg AESP (h). In rat treated with 1500 mg/kg AESP, the foot process is still a bit large; but less when compared to the untreated-SHR group (d).

DISCUSSION

Herbal supplements have become more commonly used recently due to their lower side effects in the protection and treatment of certain diseases. In this study, we determined the phytochemical constituents of the aqueous extract of *S. polyanthum* (AESP) and the nephroprotective effects in the hypertensive animal model. For the first time, we have shown that AESP can ameliorate hypertensive induced chronic kidney disease (CKD) and oxidative stress. In addition, CKD resulted in functional disturbances and cellular damages in the kidneys are reduced by AESP supplementation. We demonstrated various compounds in AESP, including phenolics, flavonoids and alkaloids, as shown in Table 1. Previous studies reported that ethanol extract of *S. polyanthum* contains flavonoid, tannin, triterpenoid, carbohydrate, alkaloid and steroid (Kusuma et al. 2011), while in methanol extract, tannins, glycosides, flavonoids and alkaloids have been detected (Widyawati et al. 2015). Total phenolic and flavonoid content as well as antioxidant activities were also high in AESP.

After 12 weeks of daily administration of AESP, the systolic blood pressure (SBP) decreased significantly in all AESP treated groups when compared to untreated group. In fact, SBP levels of all AESP treated groups were normalized at the end of the study, suggesting that AESP is a potential anti-hypertensive agent. Importantly, it was reported that aqueous and methanolic extracts of *S. polyanthum* leaves significantly lowered blood pressure in spontaneously hypertensive rats (SHR) when administered intravenously (Ismail et al. 2013), which support the present study. There is considerable evidence supporting the view that dietary intake of antioxidants and polyphenols may have an effect of primary prevention or reduction of

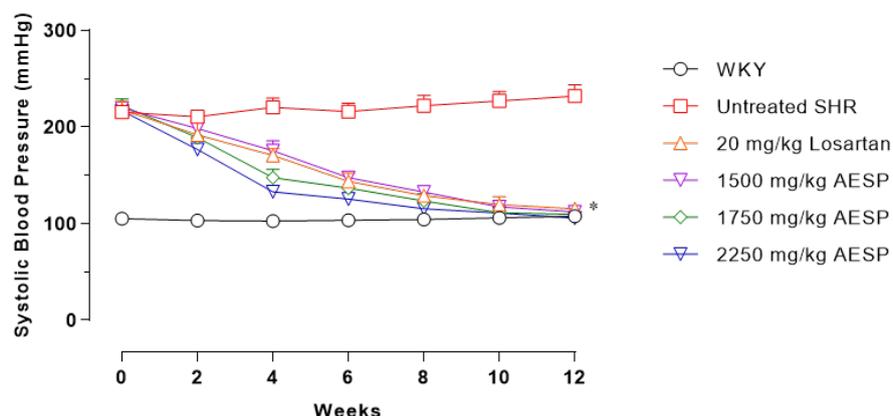


FIGURE 2. Effects of aqueous extract of *S. polyanthum* leaves (AESP) on systolic blood pressure in SHR during the treatment period of 12 weeks. Each group consisted of 10 rats (n=10). WKY, Wistar-Kyoto rats; Losartan treated, SHR treated with Losartan 20 mg/kg; AESP 1500 mg/kg, SHR treated with AESP 1500 mg/kg; AESP 1750 mg/kg, SHR treated with AESP 1750 mg/kg; AESP 2250 mg/kg, SHR treated with AESP 2500 mg/kg. * $p < 0.001$ compared to untreated SHR.

TABLE 3. The effects of AESP on renal parameters

| Group | BUN (mmol/L) | Cr (μ mol/L) | UA (μ mol/L) | K (mmol/L) | Na (mmol/L) |
|------------------|-----------------|-------------------|-------------------|----------------|--------------|
| WKY | 6.80 (0.70) | 44.02 (2.47) | 111.1 (33.79) | 5.7 (0.35) | 140.6 (0.89) |
| Untreated SHR | 10.97*** (0.80) | 56.10*** (5.95) | 278.1*** (66.70) | 6.52* (0.63) | 139.4 (1.68) |
| Losartan treated | 9.13**.# (1.28) | 43.98### (3.74) | 107.7### (15.44) | 5.82 (0.35) | 140.2 (1.79) |
| AESP 1500 mg/kg | 7.35### (0.96) | 39.42### (1.17) | 119.6### (14.04) | 5.78 (0.33) | 139.0 (1.00) |
| AESP 1750 mg/kg | 7.72### (0.87) | 48.77# (2.69) | 100.5### (14.28) | 5.36### (0.36) | 139.2 (0.84) |
| AESP 2250 mg/kg | 7.86### (0.48) | 48.56# (1.73) | 83.72### (20.29) | 5.22### (0.15) | 140.0 (2.34) |

Note: Data are presented as mean (with standard deviation), n=10 per group. WKY, Wistar-Kyoto rats; Losartan treated, SHR treated with Losartan 20 mg/kg; AESP 1500 mg/kg, SHR treated with AESP 1500 mg/kg; AESP 1750 mg/kg, SHR treated with AESP 1750 mg/kg; AESP 2250 mg/kg, SHR treated with AESP 2500 mg/kg. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to WKY; # $p < 0.05$, # $p < 0.01$ and ### $p < 0.001$ compared to untreated SHR (one way ANOVA followed by Tukey's post-hoc test).

TABLE 4. The effects of AESP on oxidative stress markers

| Group | MDA (nmol/mg Protein) | TAC (μ M Trolox Equivalents) | SOD (U/mg Protein) | CAT (U/mg Protein) |
|------------------|-----------------------|-----------------------------------|--------------------|--------------------|
| WKY | 5.50 (0.71) | 233.5 (50.55) | 2.83 (0.38) | 13.84 (0.11) |
| Untreated SHR | 13.15*** (1.54) | 172.5 (7.69) | 0.57*** (0.25) | 13.93 (0.09) |
| Losartan treated | 6.56### (1.02) | 206.9 (14.50) | 3.00### (0.21) | 13.81 (0.18) |
| AESP 1500 mg/kg | 8.65# (0.51) | 219.2 (17.49) | 2.21### (0.51) | 13.85 (0.05) |
| AESP 1750 mg/kg | 8.85# (0.66) | 233.0 (43.83) | 2.94### (0.21) | 13.82 (0.17) |
| AESP 2250 mg/kg | 6.33### (1.35) | 225.1 (22.78) | 1.24** (0.07) | 13.94 (0.05) |

Note: Data are presented as mean (with standard deviation), n=10 per group. WKY, Wistar-Kyoto rats; Losartan treated, SHR treated with Losartan 20 mg/kg; AESP 1500 mg/kg, SHR treated with AESP 1500 mg/kg; AESP 1750 mg/kg, SHR treated with AESP 1750 mg/kg; AESP 2250 mg/kg, SHR treated with AESP 2500 mg/kg. ** $p < 0.01$ and *** $p < 0.001$ compared to WKY; # $p < 0.05$, ### $p < 0.001$ compared to untreated SHR (one-way ANOVA followed by Tukey's post-hoc test).

TABLE 5. The effects of AESP on full blood count

| Group | Total RBC ($\times 10^{12}/L$) | Hemoglobin (gm/L) | Packed Cell Volume (L) | Total WBC ($\times 10^9/L$) | Lymphocyte (%) | Platelet Counts ($\times 10^9/L$) |
|------------------|-------------------------------------|----------------------|---------------------------|----------------------------------|----------------|--|
| WKY | 8.67 (0.17) | 151.0 (2.08) | 0.47 (0.01) | 7.30 (0.23) | 57.7 (0.33) | 685.7 (5.67) |
| Untreated SHR | 9.23 (0.12) | 145.7 (2.40) | 0.46 (0.01) | 6.67 (0.72) | 66.0 (2.00) | 868.7 (26.59) |
| Losartan treated | 9.05 (0.09) | 144.7 (1.20) | 0.46 (0.01) | 6.90 (0.21) | 54.3 (2.60) | 827.0 (31.10) |
| AESP 1500 mg/kg | 9.47 (0.09) | 151.7 (0.33) | 0.47 (0.01) | 7.13 (0.22) | 58.0 (6.66) | 766.3 (23.56) |
| AESP 1750 mg/kg | 8.73 (0.27) | 141.7 (1.67) | 0.44 (0.01) | 7.47 (0.64) | 63.3 (3.28) | 803.7 (59.83) |
| AESP 2250 mg/kg | 9.03 (0.09) | 143.0 (1.73) | 0.45 (0.01) | 7.47 (0.26) | 64.3 (2.33) | 780.3 (78.67) |

Note: Data are presented as mean (with standard deviation), n=10 per group. WKY, Wistar-Kyoto rats; Losartan treated, SHR treated with Losartan 20 mg/kg; AESP 1500 mg/kg, SHR treated with AESP 1500 mg/kg; AESP 1750 mg/kg, SHR treated with AESP 1750 mg/kg; AESP 2250 mg/kg, SHR treated with AESP 2500 mg/kg (one-way ANOVA followed by Tukey's post hoc test).

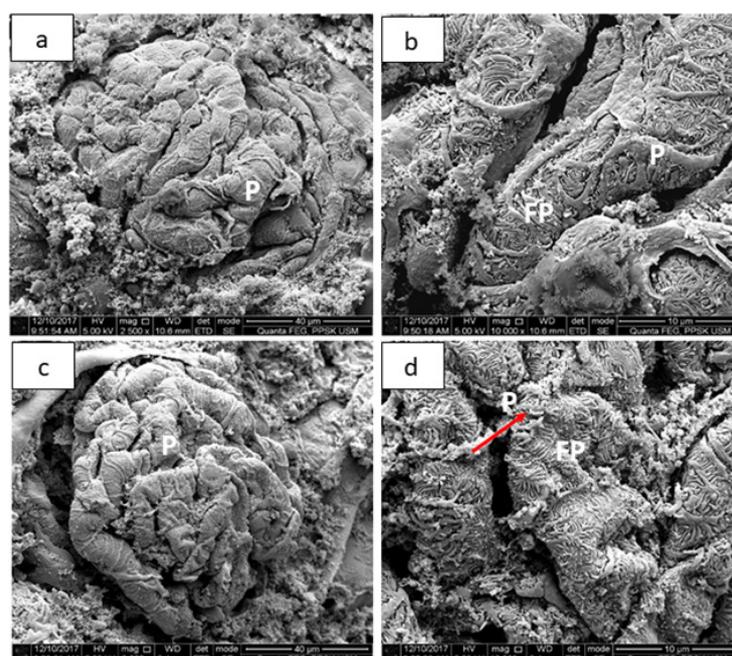


FIGURE 3. Scanning electron microscopic images of the interior of an adult rat glomerulus showing interdigitating foot processes (FP) encompassing capillary loops and (P) podocyte cell body from WKY (a-b) and untreated SHR (c-d) under 2500x and 10000x respectively. In WKY rat, a regular arrangement with parallel, longer and thin podocytes are seen. In SHR rat, the podocytes appear large in size and blunted, the foot process is irregular in thickness and some started to detach from the capillary loop. There is also loose podocyte seen (red arrow).

hypertension (Redon et al. 2003), which further support AESP is potential to reduce blood pressure.

Kidney disease is considered to be a cause and consequence of hypertension. In the present study, it is proposed that the CKD is a consequence of hypertension of at least for 12 weeks, which is considered as chronic duration (Mohammed Ali et al. 2017). Although systemic hypertension is linked to glomerular hypertension, the exact mechanism by which glomerular hypertension induces glomerular injury is not clear. The features of CKD are well demonstrated by the biochemical and histopathological changes in untreated SHR group. The

abnormal biochemical changes include increase in serum concentrations of creatinine, urea-nitrogen and uric acid indicated a decline in kidney function (Mohamadi Yarijani et al. 2019). Reduction of renal function in CKD is may be due to glomerular hypertrophy (Varatharajan et al. 2017), mesangial cells proliferation and their contraction (Martinez-Salgado et al. 2007), and increased resistance of renal vessels (Randjelovic et al. 2017). Kidney expresses its own renin-angiotensin-aldosterone system (RAAS) and inappropriate intraglomerular RAAS activation may decrease the renal function, such as in hypertensive nephropathy, diabetic nephropathy and glomerulonephritis

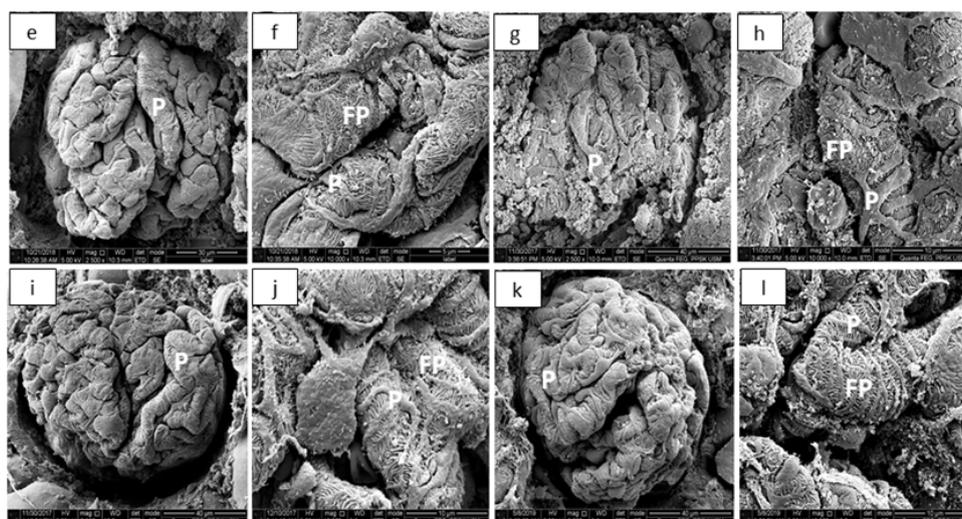


FIGURE 4. Scanning electron microscopic images of the interior of an adult rat glomerulus showing interdigitating foot processes (FP) encompassing capillary loops and (P) podocyte cell body from Losartan treated (e-f), AESP 1500mg/kg (g-h), AESP 1750 mg/kg (i-j) and AESP 2250 mg/kg (k-l) under 2500x and 10000x respectively. There are marked improvement of the podocytes, which appear parallel, longer and thin in size. However, in 1500 mg/kg AESP group, the foot process is still a bit large (h).

(Siragy & Carey 2010). Interestingly, the levels of renal parameters were improved in all SHR treated groups, Losartan and AESP, with exception of potassium levels which reduced significantly only in AESP 1750 mg/kg and 2250 mg/kg groups.

The precise mechanisms for hypertension and associated renal damage are not well clarified. In order to further assess the possible underlying mechanism of CKD in hypertension, we measured oxidative stress markers. Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense systems, and it is believed to be involved in many types of disease processes. In people with hypertension, ROS are elevated, while antioxidant capacity is decreased and many of which develop end-stage renal disease (ESRD) (Manning et al. 2005). ROS are harmful to cells and tissues, thus a proper balance between ROS production and removal is required to maintain normal biological conditions (Honda et al. 2019). Increased ROS production decreased nitric oxide (NO) levels and antioxidant bioavailability have been reported in hypertensive patients and various animal models of hypertension (Redon et al. 2003; Tanito et al. 2004). In the vascular wall, ROS is primarily derived from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that stimulated by hormones, including angiotensin II (AT-II) and endothelin-1 (ET-1), and ROS is also generated by increased mechanical stimuli on the vascular wall (Rodrigo & Rivera 2002).

Evidence suggests that there is a decrease in enzymatic antioxidant in hypertensive humans and animal models of hypertension, resulting in reduced antioxidant defenses

(Zhou et al. 2006; Lee et al. 2010). For example, a reduction in superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities have been demonstrated in newly diagnosed and untreated hypertension that is inversely correlated with blood pressure (Baradaran et al. 2014). Increased levels of MDA and decreased SOD, catalase (CAT) and glutathione peroxidase GPX were detected in hypertensive patients compared to normal subjects (Ahmad et al. 2013). In untreated SHR, there were increased serum levels of malondialdehyde (MDA), a byproduct derived from lipid peroxidation. In addition, the levels of SOD were significantly lower in this group, which further support oxidative stress as a possible cause of kidney disease in this study, which is consistent with previous study (Manning et al. 2005). In AESP treated groups, MDA levels were significantly lower compared to untreated SHR, and therefore AESP was able to ameliorate oxidative stress, which is comparable to Losartan. Significant increased SOD has been observed in AESP treated groups that may be attributed to lower levels of MDA in these groups. However, AESP at 2250 mg/kg did not increase the activity of SOD, in fact the level was significantly lower compared to WKY group.

The improvement in oxidant/antioxidant status may be attributed to its phytochemical compounds, gallic acid as demonstrated by the HPLC analysis. The therapeutic effects of gallic acid and its derivatives have been implicated in various diseases due to its biological and pharmacological activities, including antioxidant, antimicrobial, and anti-inflammatory (Kahkeshani et al. 2019). In rat models of myocardial infarction, pretreatment with gallic acid increases the activity of antioxidant enzymes, such as SOD,

CAT, glutathione transferase (GST), and GPx, also non-enzymatic antioxidant agents, such as glutathione (GSH), vitamin C, and vitamin E (Priscilla et al. 2009).

In scanning electron microscopy study, renal histology in untreated SHR revealed features of renal damage, including distorted and shrunk glomerulus with irregular thickness of the foot process (Ramli et al. 2017). The main pathological changes associated with chronic kidney disease include glomerulosclerosis and tubulointerstitial fibrosis, which result in a loss of normal renal architecture (Mullins et al. 2016). In the present study, we demonstrated that 12 weeks of hypertension induced renal damage. Renal damage secondary to hypertension is usually affects the small renal arteries of the glomerulus (Mule et al. 2008) and the degree of the damage increases with the duration of hypertension (Wang et al. 2013). In hypertensive renal damage, the capillary plexus collapses and the basement membrane shrinks, leading to renal ischemia and glomerular ischemia, which demonstrated as a ball sclerosis, the glomerular sclerosis (Wang et al. 2013). Study in mice showed a larger glomerular size with widened and effaced foot processes in hypertensive group (Li et al. 2019). Podocytes, the terminally differentiated cells of glomerular filtration barrier, play an active role in preventing plasma proteins from entering the urinary ultrafiltrate. In this study, the podocytes of untreated SHR were large in size and blunted. Podocytes are the main targets of various soluble and cellular products, including ROS, toxins, complements and antibodies (Reiser & Altintas 2016) that may support the podocytes changes in this study.

Meanwhile, foot processes interdigitate with foot processes from adjacent podocytes to form a network of narrow and uniform gaps. When podocytes are injured, the elaborate structure of podocytes is physically altered, i.e. 'foot process effacement', which is found in many proteinuric kidney diseases (Reiser & Altintas 2016). In the present study, the foot process in the untreated SHR group was irregular in thickness and some started to detach from the capillary loop. Interestingly, there were marked improvement of the glomerular, the Bowman's space and podocytes appearance in SHR treated with Losartan and AESP at 1750 mg/kg and 2250 mg/kg. However, in 1500 mg/kg AESP group, although the glomerular and the Bowman's space appeared normal, the foot process is still a bit large; but less when compared to the untreated-SHR group. Previously, we demonstrated that 4 weeks administration of 2000 mg/kg methanolic extract of *S. polyanthum* (MESP) leaves to SHR rats improved the histological changes of renal structure when compared to untreated SHR (Ramli et al. 2017).

Appropriate anti-hypertensive agent reduces the abnormalities of renal outcome in kidney disease (Judd &

Calhoun 2015). For example, the use of Angiotensin II type 1 receptor (AT1R) antagonist (Losartan) on SHR rats with ischemic acute renal failure (ARF) have been demonstrated to improve blood pressure, renal function and structure (Ivanov et al. 2014). Treatment with Losartan induced a significant increase of creatinine and urea clearance, and reduced cortico-medullary necrosis and tubular dilatation in the kidney when compared to untreated SHR with ARF. Preventing hypertensive induced CKD is necessary as no symptoms shown at early stage of renal damage, the patients usually have no symptoms (Wang et al. 2013). Furthermore, unlike acute kidney injury, the damage that occurs in the chronic kidney disease is irreversible, thus preventing disease progression and preserving organ function is extremely important (Mohammed Ali et al. 2017). The values of hematological parameters were normal and the improvement in the biochemical parameters suggested that AESP does not toxic to the body. It was reported previously that administration of ethanol extract of *S. polyanthum* did not show toxicity on the hematological parameters, renal and liver functions (Sumiwi et al. 2019). The findings of this study may indicate the potential mechanism of AESP as a nephroprotective effects in hypertension.

CONCLUSION

Administration of AESP significantly normalized SBP and improved biochemical parameters. Although all the AESP treated groups significantly reduced MDA levels, only AESP at 1500 mg/kg and 1750 mg/kg were able to significantly increase SOD levels compared to untreated SHR. Both AESP at 1750 mg/kg and 2250 mg/kg significantly improved all histological changes in renal damage. It is therefore suggested that AESP has anti-hypertensive and nephroprotective effects, possibly partly due to its phenolic compounds that have high antioxidant properties. However, further studies are needed to determine the exact molecular mechanisms of action that involve in anti-hypertensive and nephroprotective effects.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Liza Noordin
Department of Physiology, School of Medical Sciences,
Universiti Sains Malaysia, Health Campus, 16150
Kubang Kerian, Kelantan, Malaysia.

Nurul Syahida Ramli
Biomedicine Programme, School of Health Sciences,
Universiti Sains Malaysia, Health Campus, 16150
Kubang Kerian, Kelantan, Malaysia.

Nor Hidayah Abu Bakar
Faculty of Medicine, Universiti Sultan Zainal Abidin,
Medical Campus, 20400 Kuala Terengganu, Terengganu,
Malaysia.

Corresponding Author: Wan Amir Nizam Wan Ahmad
Biomedicine Programme, School of Health Sciences,
Universiti Sains Malaysia, Health Campus, 16150
Kubang Kerian, Kelantan, Malaysia.

Corresponding author email: wanamir@usm.my