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Original article

Peperomia pellucida (L.) Kunth as an angiotensin-converting enzyme inhibitor in two-kidney, one-clip Goldblatt hypertensive rats

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ABSTRACT

Backgroud: Peperomia pellucida (L.) Kunth has been used widely to treat headache, kidney disease, fever, and hypertension. Previous *in vitro* studies discovered that the flavonoid-rich extract of this plant has potential hypotensive effects, specifically angiotensin-converting enzyme (ACE)-inhibitory activity. However, there is insufficient scientific evidence to validate the result *in vivo*.
Purpose: This study investigated the dose dependencies of the effects of the ethyl acetate fraction of the ethanolic extract of this plant on blood pressure and biomarkers associated with the renin–angiotensin–aldosterone systems (RAAS), such as angiotensin II (AII) and the plasma renin concentration (PRC).
Study design: In total, 30 two-kidney, one-clip (2K1C) hypertensive model rats were divided into five groups (n = 6 each): model group, captopril 25 mg/kg BW group, and three different ethyl acetate groups (25, 50, and 100 mg/kg BW). Another six rats comprised the sham group.
Methods: Renal hypertensive rats (RHRs) were generated using stainless steel modification clips. Drugs were administered via oral gavage for 2 consecutive weeks. Blood pressure was measured weekly prior to treatment. Blood samples were collected before treatment and after the last dose to measure AII and PRC. The left kidney was isolated for histopathological examination.
Results: Blood pressure, AII levels, and PRC were elevated after 6 weeks in RHRs. Treatment with captopril and the ethyl acetate fraction of *P. pellucida* (L.) Kunth decreased blood pressure, AII levels, and PRC. The ethyl acetate fraction at a dose of 50 mg/kg BW had similar ACE-inhibitory effects as captopril. Histopathological examination disclosed coagulative necrosis in clipped kidneys. Impairment was alleviated in a dose-dependent manner by *P. pellucida* (L.) Kunth, similarly as observed in the captopril group.
Conclusion: P. pellucida (L.) Kunth targets the renin–angiotensin–aldosterone system, which might explain its antihypertensive effects.
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1. Introduction

Hypertension is one of the main risk factors associated with cardiovascular disease (Mills et al., 2016; Franklin and Wong, 2013). It is known that hypertension affects 25% of the world's adult population, and this percentage is likely to increase to 29% by 2025 (Mittal and Singh, 2010). The renin-angiotensin-aldosteron system (RAAS) is one of the systems involved in regulating blood pressure, which affects various vascular responses (Manrique et al., 2009). RAAS overactivation increases angiotensin II (AII) resulting from the conversion of angiotensinogen by renin and angiotensin-converting enzyme (ACE) (Santos et al., 2019). AII affects the heart, kidneys, and vasculature through its action on different receptors. The high level of AII will increase blood pressure by

Abbreviations: ACE, angiotensin-converting enzyme; RAAS, the renin–angiotensin–aldosterone system; AII, angiotensin II; DBP, diastolic blood pressure; PPF, *Peperomia pellucida* fraction; PRC, plasma renin concentration; RHR, renal hypertensive rat; SBP, systolic blood pressure; 2K1C, two-kidney one-clip.

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vasoconstriction, increasing reabsorption of sodium, water retention, and blood volume, resulting in hypertension (Ferrari, 2013). ACE to be one of the therapeutic targets for controlling high blood pressure because of its essential roles in the maintenance of electrolyte balance and arterial blood pressure through the renin–angiotensin–aldosterone system (Bernstein et al., 2014; Romero, 2007; Coates, 2003).

One of the plants used as an alternative remedy for hypertension is *Peperomia pellucida* (L.) Kunth. This fleshy annual herb belongs to the Piperaceae family. Its common names are pepper elder, shiny bush, rat-ear, and man-to-man, and it is primarily found in tropical areas (de Fatima et al., 2004; Aziba et al., 2001; Khan et al., 2008; Raghavendra and Kekuda, 2018). The plant has been used traditionally as a diuretic and cholesterol-lowering therapy in the treatment of hypertension and kidney disorders (de Fatima et al., 2004; Aziba et al., 2001). From a previous study, this plant reported has antipyretic activity (Khan et al., 2008), analgesic activity (Aziba et al., 2001), anti-inflammatory properties (de Fatima et al., 2004), anticancer, antimicrobial, and antioxidant (Wei et al., 2011). The phytochemicals present in the plant include alkaloids, flavonoids, cardiac glycosides, tannins, and anthraquinones (Aziba et al., 2001; Xu et al., 2006; Aqil et al., 1993; Aqil et al., 1994; Egwuche et al., 2011; Raghavendra and Kekuda, 2018). Our previous studies illustrated that the methanolic extract of *P. pellucida* (L.) Kunth inhibited ACE activity *in vitro* with an IC_{50} of 7.17 μg/mL (Saputri et al., 2015). Another study discovered that the ethanolic extract of this herb has ACE-inhibitory also has *in vitro* effects (Mun'im et al., 2017). To extend previous findings, the current study examined the effects of the ethyl acetate fraction of *P. pellucida* (L.) Kunth (PPF) on ACE activity in renal hypertensive rats (RHRs).

2. Materials and methods

2.1. Drug and chemicals

Ethanol, methanol, dichloromethane, n-hexane, and ethyl acetate were purchased from Merck (Darmstadt, Germany). Saline solution was obtained from Sigma-Aldrich (St. Louis, MO., USA). Captopril was obtained from Dexa Medica (Jakarta, Indonesia). All solvents used in this research were analytical grade.

2.2. Plant samples and collection

The aerial components of *P. pellucida* (L.) Kunth were collected from Bogor Botanical Gardens (West Java, Indonesia) between October and December 2014. The voucher specimen of the plant material was deposited and authenticated by a botanist from the Indonesian Institutes of Sciences, Research Center for Biology (West Java, Indonesia) under the voucher specimen number 237/IPH.3.02/KS/I/2014.

2.3. Preparation of PPF

The powdered aerial parts of *P. pellucida* (4600 g) were macerated with 40 L of ethanol:water (80:20) at room temperature for 48 h. The extract was filtered, and then the residue was extracted using the same solvent. The extraction process was repeated three times. The collected ethanolic extracts were evaporated using a rotary vacuum evaporator at 55 °C to obtain a crude extract (830.2 g). The crude extract was subsequently dispersed in 500 mL of warm distilled water and shaken until homogeneous. Then, fractionation was performed by sequentially adding 500 mL each of n-hexane, dichloromethane, and ethyl acetate based on polarity and partitioning the sample using a separatory funnel, thus generating PPF (20.75 g; 2.5%). PPF was stored at 4 °C until further use.

2.4. Determination of the total flavonoid content of PPF

The total flavonoid content was determined using the method described by Azmi et al. (2019). Briefly, a working solution was created by adding plant extract equivalent to 200 mg of aqueous extract to 20 mL of acetone, 1 mL of 0.5% hexamethylenetetramine, and 2 mL of 25% HCl. The suspension was then refluxed for 30 min and filtered. Next, 20 mL of the filtrate were extracted with ethyl acetate three times, and this fraction was collected as a working solution. The blank solution was created by adding glacial acetic acid to 10 mL of working solution. The sample solution was prepared by adding 1 mL of 2% aluminum trichloride and glacial acetic acid to 10 mL of the working solution. The total flavonoid content of PPF was 2.91% (w/w).

2.5. Analysis of PPF

A portion of PPF was subjected to column chromatography for fractionation using a gradient elution of n-hexane, ethanol, and methanol to obtain several fractions. The fractions with the same chromatographic profiles were combined and fractionated several times to obtain a nearly pure fraction. Following further process, a compound was successfully obtained using preparative TLC on silica gel GF254 (ethanol:methanol, 85:15). The structure of the compound was determined using spectroscopic methods, and it was reported in our previous study as 3′,4′-dihydroxy-3-5-dimethoxy flavone-7-*O*-β-rhamnose (Kurniawan et al., 2016).

2.6. Animals

Thirty-six male Sprague–Dawley rats weighing 180–200 g were purchased from the Animal Laboratory of Bogor Agricultural Institute, Indonesia. The animals were kept under constant temperature and lighting for 2 weeks before treatment for acclimatization. The rats had free access to food and water throughout the study period. The rats were placed individually in separate cages. The study was conducted in accordance with ethical protocol reviewed and approved by Health Research Ethics Committee at Faculty of Medicine, Universitas Indonesia (Ethical Approval Number: 539/UN2.F1/ETIK/2014)

2.7. 2K1C RHR model

After acclimatization, the 2K1C hypertension model was used to create RHRs as described previously by Nurfaradilla et al. (2019). Briefly, 30 rats were anesthetized with ketamine (130 mg/kg body weight [BW], i.m.). The left posterior side of the rats was shaved and sterilized with 70% ethanol. An incision (approximately 2 cm) was made immediately below the ribs. The left kidney was pulled out to display the renal artery. Stainless steel modification clips (ID: 0.20 mm, Department of Mechanical Engineering Universitas Indonesia), as presented in Fig. 1, were placed on the left renal artery adjacent to the aorta. The incision on the skin was sutured with 3–0 sterile surgical PLAIN Catgut thread. The rats were observed for 6 weeks to permit the development of hypertension. During this time, blood pressure was measured weekly using a CODA noninvasive Tail-cuff Blood Pressure System (Kent Scientific, Torrington, CT, USA). By the end of the observation period, only animals exhibiting definite hypertension (systolic blood pressure [SBP] > 150 mmHg) were selected for further treatment. The remaining six rats underwent sham operation (normal control).

Fig. 1. Modification clip.

2.8. Grouping and administration

The 30 RHRs were randomly and equally divided into five groups and treated accordingly for another 2 weeks. The groups included the untreated RHR group, captopril (25 mg/kg BW) group, and three groups treated with 25 (low), 50 (medium), or 100 mg/kg BW (high) PFF. PPF and captopril were dispersed in 0.5% CMC homogenously prior to treatment. Treatment was administered daily at 9–10 am for 2 weeks. The rats were weighed daily, and the dose of the drug was adjusted accordingly.

2.9. Blood pressure measurement

SBP and diastolic blood pressure (DBP) were measured weekly by the tail-cuff method using a CODA noninvasive Tail-cuff Blood Pressure System (Kent Scientific, Torrington, CT, USA). The animals were placed in a restrainer and acclimated to the chamber at 30 °C for 15–30 min prior to experimentation to ensure reproducible blood pressure measurements (4–5 measurements/animal/session) (Nurfaradilla et al., 2019). All blood pressure measurements were performed at the same time.

2.10. Plasma and organ sample collection

After the blood pressure measurement at the end of experiment, the rats were intramuscularly anesthetized with 130 mg/kg BW ketamine. Bloods samples were collected from the abdominal aorta using heparinized tubes. Plasma was separated by centrifugation at 1000 × *g* for 15 min at 2 °C-8 °C (Hettich Zentrifugen Micro 200R, Germany). The plasma samples were stored at − 20 °C for further biochemical analysis. Both kidneys were isolated immediately, rinsed with normal saline, and fixed in 10% formalin for histopathological examination.

2.11. Biochemical analysis

The AII concentration and PRC were measured using a sandwich enzyme immunosorbent assay-based kit (ELISA). Plasma AII levels were measured using a Cusabio ANG-II Elisa Kit (no. CSB-04494r, Cusabio, Wuhan, China), whereas PRC was estimated using a Cusabio Rat Renin Elisa Kit (no. CSB-E08702t, Cusabio, Wuhan, China). The ELISA analysis of all parameters was done according to the instruction manual from manufacture. The samples were measured using a 96 well plate reader, The Glomax®-Multi Detection System (Promega, USA) at a wavelength of 450 nm.

2.12. Histopathology

Disease progression was examined via histological examination. A portion of the organ was removed, washed with normal saline, and fixed in 10% formalin solution. After fixation, the tissues were embedded in paraffin wax. The solid section of each tissue was cut using microtome at a thickness of and then stained with hematoxylin–eosin solution. The section was observed under a light microscope for histological changes.

2.13. Statistical analysis

The data were presented as the mean ± SD. The results were processed using SPSS 17.0 statistics software. Statistical analysis was performed using one-way ANOVA followed by a post hoc test. $P < 0.05$ indicated statistical significance.

3. Result

3.1. Weight

The rats gained weight regularly during the experiment, illustrating that the induction of hypertension did not result in weight loss (Table 1). The mean percent weight gain values in the sham, model, captopril, PPF low, PPF medium, and PPF high groups were 7.88, 2.27, 3.55, 7.40, 8.56, and 3.32%, respectively.

3.2. SBP and DBP

Placing a clip in one renal artery induced gradual increases of SBP and DBP. After 6 weeks, blood pressure reached 160/100 mmHg (Fig. 2). Treatment with captopril and PFF reduced both SBP and DBP, although the values did not resemble the control data. The medium dose of PPF had similar effects on blood pressure as captopril (Tables 2–3).

3.3. AII levels and PRC

The AII concentration and PRC were significantly higher in RHRs than in sham-operated rats. Treatment with captopril and PPF partially reversed both changes. Specifically, the AII concentration was similar between the PFF medium and captopril groups (Table 4).

3.4. Histopathology

Based on the histological examination, coagulative necrosis was observed in RHRs, and the evidence of impairment was lessened by treatment with captopril, as well as PPF in a dose-dependent manner (Fig. 3).

4. Discussion

The objective of this study was to determine the efficacy of PPF on the elevation of SBP, DBP, plasma AII levels, and PRC in 2K1C rats. The 2K1C model was first described by Goldblatt and colleagues (Goldblatt et al., 1934). The original 2K1C technique was performed to reduce the diameter of renal arteries in dogs and monkeys via the placement of silver clips of various sizes. Hypertension was induced achieved in animals by clipping the renal artery using adjustable silver clips (Helle et al., 2006). These clips have certain disadvantages, such as variance in flexibility, which leads to poor fabrication, and differences in availability from manufacturers. Therefore, this study aimed to induce renal hypertension by placing clips fabricated from cheap and easy-to-obtain material, namely stainless steel clips with an internal diameter of

Table 1
Changes in the weights of renal hypertensive rats (RHRs) over time before and after treatment (n = 6 per group).

Group	Dose (mg/kg BW)	Before treatment (g)	After treatment	
			One week (g)	Two weeks (g)
Sham	–	200.0 ± 0.70	205.0 ± 0.20	217.0 ± 0.15
Model	–	1902 ± 0.74	194.9 ± 0.41	197.2 ± 0.43
Captopril	25	198.2 ± 0.43	199.9 ± 0.68	202.8 ± 0.15
PPF low	25	199.1 ± 0.09	207.8 ± 0.68	215.0 ± 0.63
PPF medium	50	192.3 ± 0.41	199.7 ± 0.19	210.3 ± 0.34
PPF high	100	192.3 ± 0.42	197.4 ± 0.47	198.9 ± 0.44

Note: Sham: no treatment; Model: untreated RHR model group; Captopril: RHR model treated with captopril (25 mg/kg BW, p.o.); PPF low: RHR model treated with 25 mg/kg BW PPF (p.o.); PPF medium: RHR model treated with 50 mg/kg BW PPF (p.o.); PPF high: RHR model treated with 100 mg/kg BW PPF (p.o.).

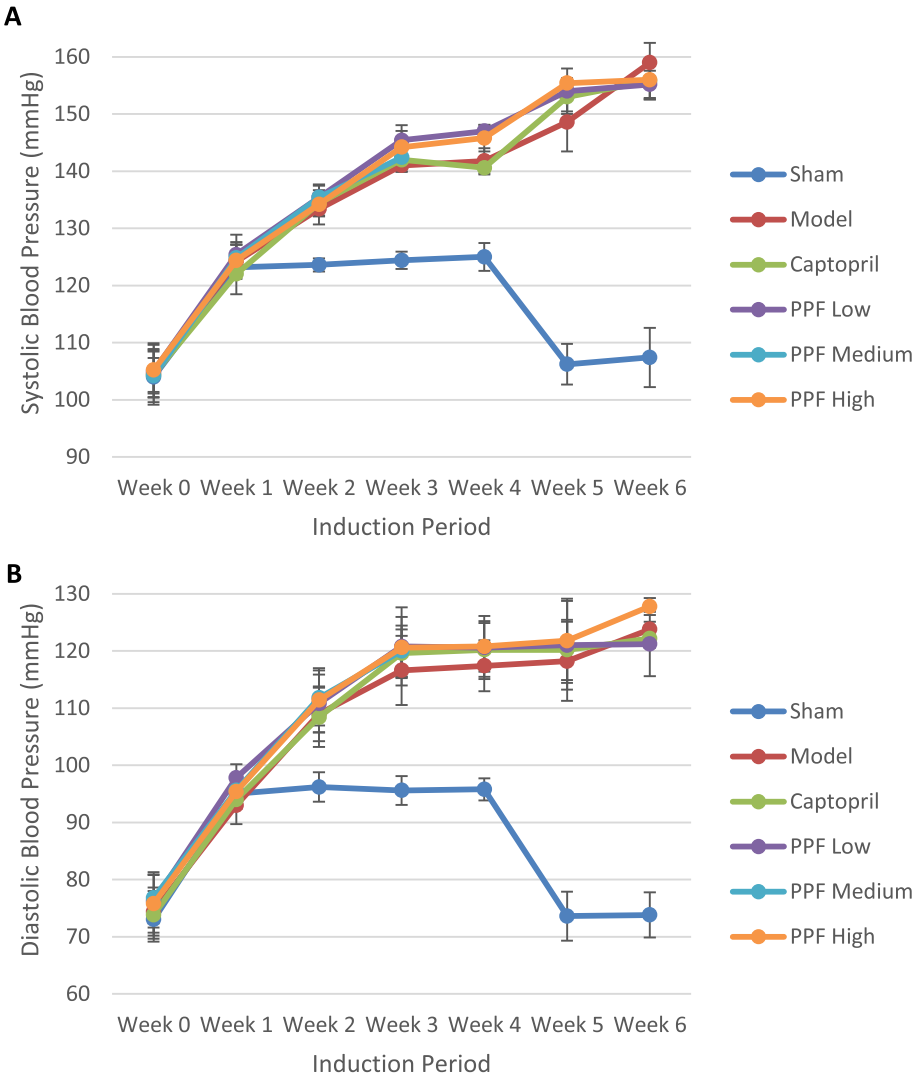

Fig. 2. Changes of systolic (A) and diastolic (B) blood pressure in the sham, model, captopril, and PPF groups. Note: Sham: no treatment; Model: untreated RHR model group; Captopril: RHR model treated with captopril (25 mg/kg BW, p.o.); PPF low: RHR model treated with 25 mg/kg BW PPF (p.o.); PPF medium: RHR model treated with 50 mg/kg BW PPF (p.o.); PPF high: RHR model treated with 100 mg/kg BW PPF (p.o.).

0.20 mm and dimensions of 3.5 × 2 × 1 mm^3. The clips had different lengths to make the clamping process easier. Other studies of induced renal hypertension used modified clips manufactured in Iran and the USA. In Iran, clips were fabricated in 2007 using solid Plexiglas (4 × 2 × 21 mm^3). Placement of these clips induced hypertension within 4 weeks (Nekooeian and Mashhoodi, 2007). In 2011, a research group in the USA induced renal hypertensive using medical-grade titanium clips (circle shaped). Application of clips with widths of 0.23, 0.25, and 0.27 mm for 9 days resulted in higher mean arterial blood pressure (Chelko et al., 2012).

In this study, stainless steel modification clips increased blood pressure within 6 weeks. In the 2K1C model, the levels of circulat-

Table 2
Systolic blood pressure in sham, model, captopril, and PPF groups before and after treatment (n = 6 per group).

Group	Dose (mg/kg BW)	Before treatment (mmHg)	After treatment (mmHg)	
			One week	Two weeks
Sham	–	107.4 ± 5.18	110.0 ± 1.41	106.6 ± 2.07
Model	–	159.0 ± 3.46*	159.6 ± 2.07*	159.0 ± 2.74*
Captopril	25	155.8 ± 3.27*	124.3 ± 2.51*#	112.2 ± 6.06*#
PPF low	25	155.2 ± 2.39*	146.0 ± 2.55*#	145.2 ± 2.77*#
PPF medium	50	155.2 ± 1.30*	136.0 ± 1.00*#	124.4 ± 1.44*#
PPF high	100	156.0 ± 3.39*	145.4 ± 2.70*#	± 3.08*#

Note: Sham: no treatment; Model: untreated RHR model group; Captopril: RHR model treated with captopril (25 mg/kg BW, p.o.); PPF low: RHR model treated with 25 mg/kg BW PPF (p.o.); PPF medium: RHR model treated with 50 mg/kg BW PPF (p.o.); PPF high: RHR model treated with 100 mg/kg BW PPF (p.o.). *p < 0.05 compared to sham; #p < 0.05 compared to model group.

Table 3
Diastolic blood pressure in the sham, model, captopril, and PPF *groups* before and after treatment (n = 6 per group).

Group	Dose (mg/kg)	Before treatment	After treatment (mmHg)	
			One week	Two weeks
Sham	–	73.8 ± 3.96	73.8 ± 2.77	72.6 ± 5.18
Model	–	123.8 ± 1.30	126.0 ± 1.00*	125.0 ± 3.08*
Captopril	25	122.2 ± 1.64	108.2 ± 1.92*#	95.0 ± 1.00*#
PPF low	25	121.2 ± 5.63	125.2 ± 1.10*#	126.0 ± 1.00*#
PPF medium	50	123.2 ± 1.30	116.0 ± 1.22*#	109.6 ± 5.68*#
PPF high	100	127.8 ± 1.48	125.0 ± 3.08*#	115.6 ± 2.70*#

Note: Sham: no treatment; Model: untreated RHR model group; Captopril: RHR model treated with captopril (25 mg/kg BW, p.o.); PPF low: RHR model treated with 25 mg/kg BW PPF (p.o.); PPF medium: RHR model treated with 50 mg/kg BW PPF (p.o.); PPF high: RHR model treated with 100 mg/kg BW PPF (p.o.). *p < 0.05 compared to sham; #p < 0.05 compared to model group.

Table 4
Plasma angiotensin II (AII) and plasma renin concentration (PRC) in sham, model, captopril and *P. pellucida* after treatment (n = 6 per group).

Group	Dose (mg/kg)	AII (pg/mL)	PRC (mU/mL)
Sham	–	34.38 ± 0.68	60.2 ± 0.98
Model	–	45.63 ± 1.68*	76.2 ± 1.10*
Captopril	25	31.38 ± 0.88*#	59.8 ± 1.67*#
PPF low	25	39.38 ± 1.68*#	68.2 ± 2.68*#
PPF medium	50	34.88 ± 1.05*#	61.0 ± 1.67*#
PPF high	100	38.38 ± 1.12*#	66.6 ± 1.79*#

Note: Sham: no treatment; Model: untreated RHR model group; Captopril: RHR model treated with captopril (25 mg/kg BW, p.o.); PPF low: RHR model treated with 25 mg/kg BW PPF (p.o.); PPF medium: RHR model treated with 50 mg/kg BW PPF (p.o.); PPF high: RHR model treated with 100 mg/kg BW PPF (p.o.). *p < 0.05 compared to sham; #p < 0.05 compared to model group.

ing renin and aldosterone, which play several roles, most notably in the early phase of hypertension, were increased due to the hyperstimulation of RAAS. Furthermore, the RAAS dysregulation triggers the sudden elevation of AII after one kidney artery is clipped (Hong et al., 2020). The loss of perfusion due to the clip induces the renin production by the kidney resulting in increased formation of AII which will raise the blood pressure (Amat et al., 2014). The 2K1C method thus generates hypertensive rats with high renin and AII levels. Contrarily, the one-kidney, one-clip method generates hypertensive rats with normal renin concentrations (Bruno et al., 2004).

The potential medicines targeting RAAS have been investigated for hypertension since RAAS plays a major role in hypertension. Herbal medicines have also been considered for hypertension, both prevention and treatment, although many antihypertensive drugs have been introduced. Herbal medicines which are known to have an ACE inhibitory mechanism in RAAS can be studied for their effects on hypertension models through the 2K1C method.

P. pellucida as an antihypertensive species is expected to decrease blood pressure and plasma AII concentrations due to its ACE inhibitory action. In this study, the most effective dose of PPF was 50 mg/kg BW. The lack of better effects at higher doses might be related to antagonistic substances in PPF. However, *P. pellucida* contains flavonoids and essential oils that may have possibly contributed to the observed effects in this study. Apigenin is a flavonoid constituent in *P. pellucida* known to prevent hypertension (Kooshki and Hoseini, 2014). The effectiveness of flavonoids as ACE inhibitors had been studied in recent decades, and these agents were proven effective in inhibiting ACE activity (Actis-goretta et al., 2003; Ojeda et al., 2010). Previous studies revealed that flavonoids could inhibit zinc metalloproteinase, which catalyzes the formation of chelate complexes in the active center site of ACE (Ende and Gebhardt, 2004). Flavonoids are excellent sources of functional antihypertensive products (Guerrero et al., 2012). The antihypertensive potential of *P. pellucida* in this study is in line with the result of another study that showed this plant has a hypotensive effect in normotensive rats (Fasola and Adeboye, 2015). Nwokocha et al. (2012) reported that *P. pellucida* has a hypotensive effect, bradycardic and vasorelaxant through the nitric oxide-dependent mechanism.

Coagulative necrosis was noted on histological observation of the clipped kidneys. The most common cause of this necrosis pattern is organ ischemia caused by the loss of arterial oxygenated blood. Regarding the rat hypertensive model used in this study, the loss of arterial oxygenated blood was attributable to clipping of the renal artery (Bruno et al., 2004). Necrosis in the clipped kidneys was associated with increased oxidative stress. Meanwhile, the upregulation and translocation of the pro-death protein BNIP3, which can induce mitochondrial dysfunction and cell death, were also observed (Fedorova, et al., 2013). Amelioration of the effects of kidney clipping in the treatment groups was associated with the ACE-inhibitory effects of captopril and PPF, resulting in the improvement of renal function, inhibition of systemic and intrarenal angiotensin II production, and attenuation of intrarenal inflammation (Efrati et al., 2012).

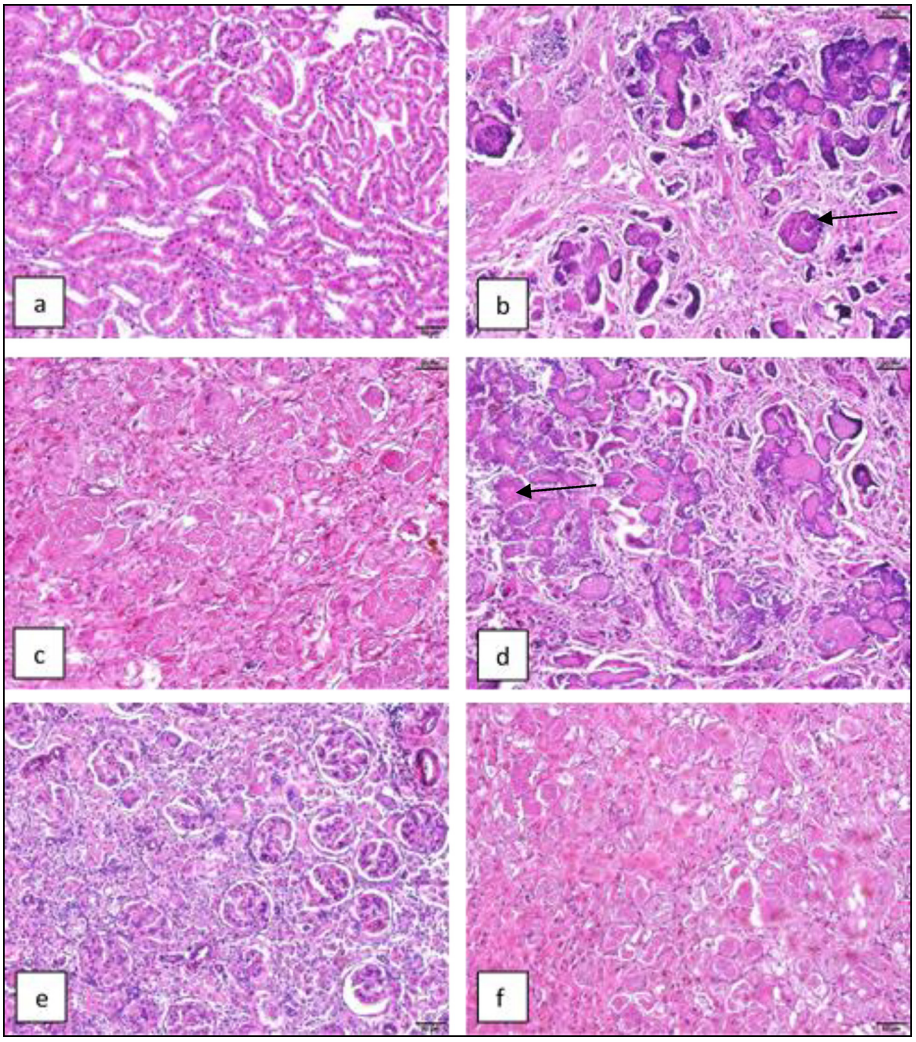

Fig. 3. Histological observation of the left kidney in 2K1C rats. a. Sham; b. Model; c. Captopril; d. PPF low; e. PPF medium; f. PPF high. → coagulative necrosis; Sham: no treatment; Model: untreated RHR model group; Captopril: RHR model treated with captopril (25 mg/kg BW, p.o.); PPF low: RHR model treated with 25 mg/kg BW PPF (p.o.); PPF medium: RHR model treated with 50 mg/kg BW PPF (p.o.); PPF high: RHR model treated with 100 mg/kg BW PPF (p.o.).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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