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Standardised flavonoid extract from *Diospyros kaki* L.f leaves alleviates high blood pressure and ventricular hypertrophy on cortisone acetate-induced hypertensive rats

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Abstract:

The current study was designed to investigate the anti-hypertensive effects of a standardised flavonoid extract from *Diospyros kaki* L.f leaves (DK extract) on cortisone acetate-induced hypertensive rats. Hypertension was induced in *Wistar* rats by subcutaneously injecting 2.5 mg cortisone acetate daily and by maintaining the rats on a 1% sodium chloride diet for a period of 28 days. The hypertensive rats were treated daily with DK extract (50 and 100 mg/kg of body weight; p.o.) or captopril (20 mg/kg of body weight; p.o.), a reference drug, for 14 consecutive days. The blood pressure and heart rate of awake rats were measured using a non-invasive tail-cuff method. The heart weight and left ventricular wall thickness of rats were measured. *In vitro* angiotensin-converting enzyme (ACE) inhibition activity of the DK extract and captopril were analysed. DK extract significantly and dose-dependently reduced systolic and diastolic blood pressure in the hypertensive rats. There were no significant changes observed in heart rate. The cortisone acetate-induced hypertensive rats had increased heart weight and left ventricular wall thickness. These actions were attenuated by the treatment of DK extract (100 mg/kg) and captopril. Moreover, DK extract inhibited ACE activity *in vitro* with an IC_{50} of 4.71±0.53 µg/ml. Our findings demonstrate that DK extract exerted anti-hypertensive effects and alleviated ventricular hypertrophy in the cortisone acetate-induced hypertensive rats and these actions occurred at least in part via ACE activity inhibition.

Keywords: **angiotensin-converting enzyme inhibitor, anti-hypertensive effect, *Diospyros kaki* L.f leaves, ventricular hypertrophy.**

Classification numbers: **3.2, 3.3**

1. Introduction

Hypertension is a medical condition characterised by persistently elevated blood pressure in the arteries. This condition is a leading cause of ischemic stroke and a major risk factor for coronary artery disease and myocardial infarction [1]. Globally, the goal is to reduce the prevalence of hypertension by 25% by 2025 [2]. To date, treatments for hypertension aim to reduce not only systolic and diastolic blood pressure but also its long-term complications, such as end-organ damage. First-line pharmacotherapy for hypertension typically involves the use of ACE inhibitors, although their efficacy may be limited in some cases because of side effects such as cough, mild skin itching, and numbness [1]. Therefore, there is growing interest in developing natural source-based antihypertensive drugs.

Persimmon (*Diospyros kaki* L.f) is a plant species belonging to the family *Ebenaceae*. This plant is widely distributed in Asian countries such as China, Japan, Korea, India, and Vietnam. Traditionally, persimmon leaves have been used for medicinal, cosmetic, and health beverage purposes. In particular, persimmon leaf tea is a popular preparation in China and Japan due to its purported ability to lower blood pressure [3]. In a previous study, we demonstrated that a standardised flavonoid extract from *Diospyros kaki* L.f leaves (DK extract) exerted antihypertensive effects in rats with two-kidney one-clip induced hypertension [4]. However, the anti-hypertensive mechanism of DK leaves and its potential effects on cardiac hypertrophy, an important long-term complication of hypertension, have not yet been clarified.

The administration of glucocorticoids together with a high-salt diet is an animal model widely used for evaluating the antihypertensive activity of drugs. This model induces volume overload hypertension in rats through the combined administration of glucocorticoids and sodium chloride. The most important advantage of this model is markedly depressed renin-angiotensin-aldosterone system activity, which enables its use in studies as an angiotensin-independent model. Furthermore, this experimental model immediately induces hypertension and cardiac hypertrophy. Therefore, it is useful for the investigation of antihypertensive effects and its complications, especially those affecting the cardiovascular system [5].

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In this study, we aim to investigate whether DK extract possesses antihypertensive effects and, if so, to elucidate the underlying mechanism. Specifically, we examined the effect of persimmon leaf extract on blood pressure and myocardial hypertrophy in cortisone acetate-induced hypertensive rats. We also assessed the ACE inhibitory activity of the persimmon leaf extract.

2. Materials and methods

2.1. Preparation of the standardized flavonoid extract from Diospyros kaki L.f leaves (DK extract)

The *Diospyros kaki* L.f leaves used in this study were collected from Lang Son Province in June 2019 and identified by Dr. Pham Thanh Huyen (Department of Medicinal Plant Resources, National Institute of Medicinal Materials, Vietnam). A voucher specimen of the herb was deposited in National Institute of Medicinal Materials. In this study, the standardised flavonoid extract was prepared as previously reported [6]. Briefly, 10 kg of the persimmon leaves were dried at 40-50^{o}C and then crushed. The dried leaves were extracted three times with 70% ethanol (10/8/8 ml/g) by refluxing for 2, 1.5, and 1 h, respectively. The collected extracts were pooled and evaporated to form a 40% alcohol concentrated extract. This ethanol extract was then adsorbed onto the column containing D101 macroporous resin. After absorption, the column was washed with 30% ethanol until the colour faded. Then, the column was eluted with 60% ethanol. After recovery of ethanol by rotary evaporation, the extract was evaporated by a water bath at 60^{o}C. The extract was dried in a vacuum oven at 50^{o}C to obtain 302 g dry extract (DK extract) with a moisture content of less than 5%.

The flavonoid contents in the DK extract were analysed as described in a previous study [6]. Briefly, the DK extract was hydrolysed in a 2-M HCl /methanol solution at 90^{o}C for 2 h. Quercetin and kaempferol contents in the hydrolysed DK extract solution were determined by HPLC (Shimazu 20A, Japan) using a Vertisep C18 column (250×4.6 mm, 5 µm) (Vertical Chromatography Co., Ltd, Bangkok, Thailand). The content of flavonoids in the DK extract was calculated as in the following formula:

Total content of flavonoids in DK extract = quercetin content × 2.55 + kaempferol content × 2.59.

According to the analysis method, the total content of flavonoids in the DK extract was calculated as 21%.

2.2. Cortisone acetate-induced hypertension and drug administration

2.2.1. Cortisone acetate-induced hypertensive model: Both male and female *Wistar* rats 8-12 weeks of age and weighing between 200 and 250 grams were provided by the Military Medical Academy, Hanoi, Vietnam. The rats were housed and maintained at a temperature of 25±1^{o}C under 65±5% humidity and a 12 h dark-light cycle (from 7:00-19:00). The rats were kept for one week to acclimatise them before the commencement of experiments. Food and water were made available for rats *ad libitum.* The present protocol of this study was approved by the Scientific Board Committee of Hanoi Medical University, Vietnam (No. 777/QD-DHYHN).

Hypertension was induced in the *Wistar* rats by daily subcutaneous injection of 2.5 mg cortisone acetate and a constant diet of 1% sodium chloride/water for a period of 28 days. Cortisone acetate was dissolved in olive oil and administered by subcutaneous injection. The rats' blood pressure and the heart rate of awake rats were measured using a non-invasive tail-cuff method [7, 8]. After 28 days of cortisone acetate administration and 1% sodium chloride diet, the blood pressure and heart rate were measured using the tail-cuff method in conscious rats. Only rats with systolic blood pressure ≥140 mmHg were used to evaluate anti-hypertensive property.

DK extract was suspended in distilled water and given to the hypertensive rats at doses of 50 and 100 mg/kg/day, p.o. Captopril (Across Organics, USA) was also dissolved in water and given to the animals at a daily dose of 20 mg/kg/day, p.o. The physiological control (sham) and the model control rats were administered tap water (Fig. 1).

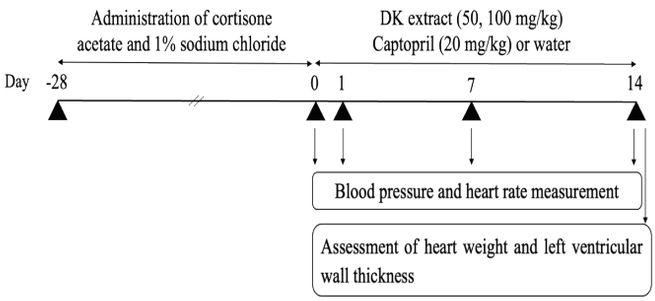

Fig. 1. Experimental schedule.

2.2.2. Measurement of the blood pressure and heart rate in awake rats: The blood pressures and heart rates of awake rats were measured using a non-invasive tail-cuff method as previously described [4]. Briefly, rats were individually placed in a warming chamber and then immobilised in a dedicated mini cage. A cuff with a pulse sensor was fixed to the tail of rats and connected to a pressure transducer. Results were recorded and analysed using Labchart Pro software (ADInstruments, Pty Ltd. Australia). Blood pressure and heart rate was measured on the first day (1 hour after the first drug was administered), and 7 and 14 days after starting the treatment. On the day of blood pressure and heart rate measurements, the rats were given medications 1 hour before the measurement (Fig. 1).

2.2.3. Assessment of heart weight and left ventricular wall thickness: After the final measurement of blood pressure on day 14, the rats were anesthetised with chloral hydrate (250 mg/kg, i.p). The heart was immediately removed and weighed, then cut into 1 mm-thick slices. Left ventricular wall thickness (9 measurements for each section) was evaluated using Image-J software (ver. 1.41, NIH; Bethesda, MD, USA) [9].

2.3. ACE inhibition assay

DK extract was dissolved in DMSO to obtain a stock solution (100 mg/ml). To determine the half-maximal inhibitory concentration (IC_{50}), a stock solution of DK extract was diluted in a borate buffer (pH 8.3) to obtain test concentrations of 1.563, 3.125, 6.250, 12.500, and 25.000 µg/ml.

Determination of the ACE inhibitory activity was conducted as described by previous studies [9-11]. Briefly, 12.5 µl of test sample (DK extract) or a reference drug, captopril (Across Organics), and a borate buffer (27.5µl) were mixed with 10 µl ACE solution (0.02 U/ml) in a 96 well-plate. This mixture was incubated at 37°C for 5 min. Then, 75 µl of N-Hippuryl-His-Leu solution (Sigma-Aldrich, USA) was added and subsequently incubated at 37°C for 30 min. The reaction was terminated using 1 M HCl. Quinoline (150 µl, Across Organics) and benzene sulfonyl chloride (75 µl, Sigma-Aldrich, USA) were added to the mixture. After diluting with 300 µl ethanol, the absorbance at 490 nm was measured using an ELISA system (EL x 808 Biotek, USA). Control samples were made similarly to the test samples with DK extract or captopril replaced by borate buffer. Each reaction was run in duplicate. ACE inhibitory activity (I%) was determined using the following formula:

$$I\,\% = (OD_{control} - OD_{test})/OD_{control} \times 100\ (\%),$$

where $OD_{control}$: absorbance value (optical density) of control sample; OD_{test}: absorbance (optical density) value of test sample.

2.4. Statistical analysis

The data were statistically analysed using SigmaPlot 12.0 (SYSTA Software Inc, Richmond, CA, USA). The results of systolic and diastolic blood pressure and heart rate were expressed as mean ± standard error of the mean (SEM). Differences between groups were assessed using two-way ANOVA followed by a Student-Newman-Keuls post hoc test for multiple comparisons. Differences of *p* value <0.05 were considered significant.

3. Results

3.1. DK extract reduced blood pressure without affecting the heart rate of the hypertensive rats

As shown in Fig. 2, the cortisone acetate-induced hypertensive model resulted in a significant development of hypertension as indicated by increases in both systolic and diastolic blood pressure when compared to the sham group ($p<0.001$). In the sham and untreated hypertensive rats, blood pressure remained stable throughout the study period. The daily administration of 50 mg/kg DK extract did not show blood pressure reducing effects one hour after the first administration compared to the vehicle-treated 2K1C rats. However, after 7 and 14 days of administration, the hypertensive rats treated with DK extract at a dose 50 mg/kg had significantly reduced blood pressure ($p<0.05$). Treatment of DK extract at a dose of 100 mg/kg significantly decreased the systolic and diastolic blood pressure of the hypertensive rats after 1 h, 7 days, and 14 days of administration compared to the control hypertensive model ($p<0.001$). Treatment with captopril at a dose of 20 mg/kg resulted in a reduction of the systolic and diastolic blood pressures that was apparent from the first administration and maintained during the following 14 days ($p<0.001$). Moreover, there was no change in heart rate across all animal groups ($p>0.05$) (Fig. 3).

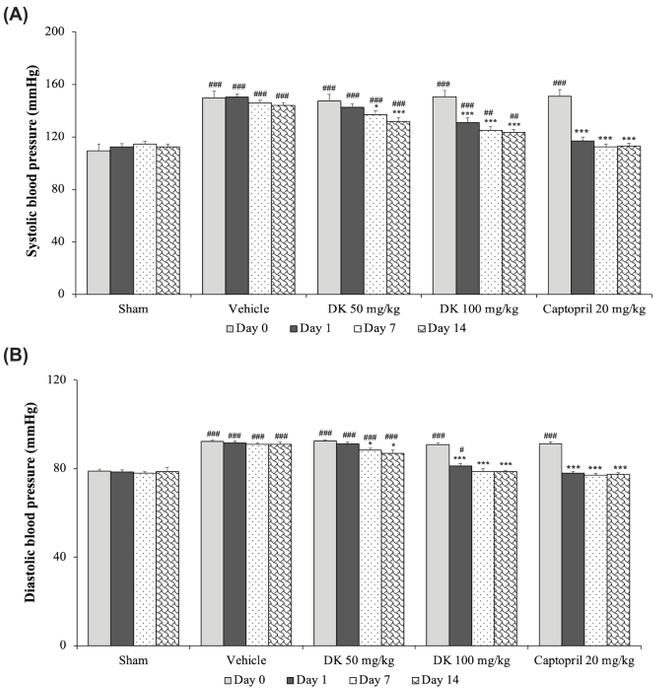

Fig. 2. Effects of DK extract on systolic and diastolic blood pressures of cortisone acetate-induced hypertensive rats: (A) systolic blood pressure and (B) diastolic blood pressure (n=10). Note: *: $p<0.05$, ***: $p<0.001$ compared to vehicle-treated hypertensive rats; ###: $p<0.001$ compared to sham animals. Data represents mean ± SEM.

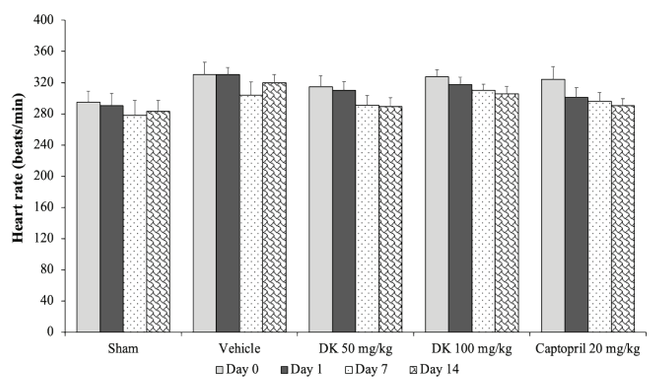

Fig. 3. Effects of DK extract on heart rate of cortisone acetate-induced hypertensive rats (n=10). Data represents mean ± SEM.

3.2. DK extract reduced ventricular hypertrophy in the hypertensive rats

Myocardial hypertrophy is a known complication of hypertension [12]. Therefore, we investigated the effects of

persimmon leaf extract (DK extract) on heart weight and left ventricular wall thickness in hypertensive rats. As shown in Table 1, cortisone acetate-induced hypertensive rats exhibited a significant increase in heart mass compared to the sham rats ($p<0.001$). However, administration of captopril (20 mg/kg) or DK extract (100 mg/kg) for 14 days led to a marked decrease in heart weight compared to the vehicle-treated hypertensive group ($p<0.05$). Additionally, treatment with captopril (20 mg/kg) or DK extract (100 mg/kg) significantly attenuated left ventricular hypertrophy in hypertensive rats. Notably, treatment with DK extract at a dose of 50 mg/kg had no significant effect on heart mass or left ventricular wall thickness in hypertensive rats (Table 1 and Fig. 4).

Table 1. Effects of DK extract on heart weight/body weight ratio and left ventricular wall thickness (n=10).

Groups	Dose (mg/kg)	Heart weight/body weight ratio (*10^3)	Left ventricular wall thickness (mm)
Sham	-	2.95 ± 0.11	2.23 ± 0.08
Vehicle-treated hypertensive rats	-	4.07 ± 0.17$^{###}$	3.29 ± 0.15$^{###}$
DK-treated hypertensive rats	50	3.64 ± 0.20$^{#}$	2.98 ± 0.12$^{###}$
	100	3.47 ± 0.15*	2.88 ± 0.09$^{###}$*
Captopril-treated hypertensive rats	20	3.24 ± 0.13**	2.79 ± 0.11$^{##}$*

*: $p<0.05$; **: $p<0.01$ compared to vehicle-treated hypertensive rat; $^{#}$: $p<0.05$; $^{##}$: $p<0.01$; $^{###}$: $p<0.001$ vs. sham animal. Data represents mean ± SEM.

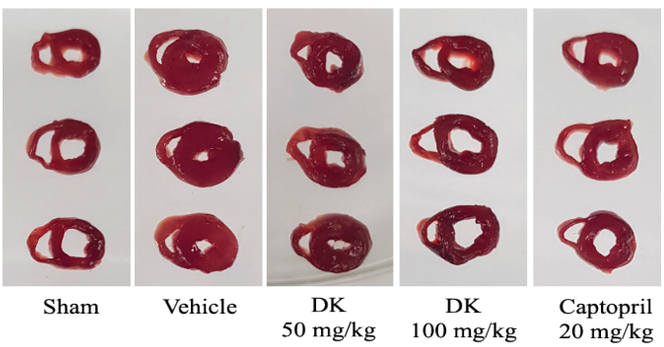

Fig. 4. Effects of DK extract on ventricular hypertrophy in the cortisone acetate-induced hypertensive rats. One mm-thick slices of heart were prepared from rats after 14 days of drug administration.

3.3. In vitro ACE inhibitory activity of DK

To investigate the possible mechanism underlying the antihypertensive effects of DK extract, we performed an ACE inhibition assay using a spectrophotometric method. The dose-response curves for ACE inhibition activity (%) in the presence of various concentrations of DK extract and captopril are shown in Fig. 5. The IC_{50} values of captopril and DK extract were calculated as 17.95±1.99 nM and 4.71±0.53 µg/ml, respectively.

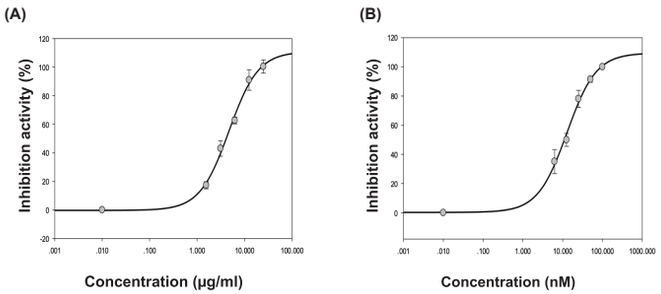

Fig. 5. *In vitro* ACE inhibitory activity of DK extract (A) and captopril (B).

4. Discussion

Hypertension is the most prevalent cardiovascular disease, and its pathophysiology in humans is multifactorial and complex. Therefore, experimental research models are used to investigate the aetiology, pathophysiology, complications, and treatment of hypertension [5]. In this study, we demonstrated that daily administration of DK extract at a dose 100 mg/kg reduced blood pressure and alleviated the left ventricular hypertrophy in cortisone acetate-induced hypertensive rats. Indeed, our findings were supported by an *in vitro* study indicating that the extract possessed ACE inhibitory activity with an IC_{50} of 4.71±0.53 µg/ml.

We employed the glucocorticoid-salt induced hypertensive model in rats to evaluate the antihypertensive effects of DK extract. This model, which combines glucocorticoid treatment and high-salt intake, induces hypertension by promoting increases in extracellular fluid volume, providing a reliable animal experimental model [3]. In a previous study, we demonstrated that a flavonoid-enriched extract from *Diospyros kaki* L.f leaves at a dose of 100 mg/kg exerted antihypertensive effects in rats with two-kidney one-clip-induced hypertension [4]. However, this renovascular hypertensive model has limitations, such as a difficulty in controlling the degree of renal damage and a large failure rate of the number of hypertensive rats. In addition, renovascular hypertension represents only a small fraction of human hypertension [13, 14]. Therefore, we conducted the glucocorticoid-salt hypertension model to evaluate the antihypertensive effects of DK extract and its underlying mechanism. Our results demonstrated that daily treatment with DK extract reduced systolic and diastolic blood pressures in a dose-dependent manner. Furthermore, the extract's antihypertensive mechanism was supported by an *in vitro* study indicating that it possessed ACE inhibitory activity with an IC_{50} of 4.71±0.53 µg/ml. The total flavonoid content in the DK extract was calculated as 21% using the HPLC method. On the other hand, it has been reported that some isolated flavonoid components from the *Diospyros kaki* leaves growing in Vietnam, such as quercetin and astragalin, reduced blood pressure *via* ACE inhibition [15-19]. Therefore, the present findings suggest that flavonoid components, through ACE inhibition activity, play a role in the anti-hypertensive effects of DK extract. In addition, quercetin decreased blood pressure by activating the Na^+-K^+-$2Cl^-$ cotransporter 1 in renal epithelial cells [20], while kaempferol enhanced relaxation of smooth muscle

cells by activating large-conductance calcium-activated potassium channels [21]. Hence, it is possible that these mechanisms also contributed to the antihypertensive effect of DK extract.

Our study also demonstrated that daily administration of DK extract at a dose of 100 mg/kg, as well as captopril at a dose of 20 mg/kg, significantly mitigated the increase in heart mass and the left ventricular wall thickness caused by hypertension. These findings provide evidence for the first time that flavonoids extracted from *Diospyros kaki* L.f leaves can reduce myocardial hypertrophy. An association between long-term hypertension and left ventricular hypertrophy has been reported [12], and preventing myocardial hypertrophy is an important endpoint in the treatment of hypertension [22]. ACE inhibitors, calcium channel blockers, beta-blockers, and diuretics have been shown to reduce left ventricular mass, while direct-acting vasodilators and alpha-adrenergic blockers do not affect myocardial hypertrophy [12]. Therefore, the suppression of left ventricular hypertrophy observed in DK-treated hypertensive rats suggests that DK extract possesses a beneficial effect on the cardiovascular system in addition to blood pressure-lowering activity.

5. Conclusions

Our study demonstrated that the daily administration of DK extract mitigated hypertension and alleviated ventricular hypertrophy in hypertensive rats, possibly through its ACE inhibition activity. These findings suggest that DK extract may be a useful treatment for hypertension and cardiovascular disease.

CRediT author statement

Thi Thanh Loan Nguyen: Investigation, Formal analysis, Writing-original draft; Thi Van Anh Pham: Conceptualisation, Interpretation of findings, Drafting the manuscript; Thi Xoan Le: Conceptualisation, Interpretation of findings, Writing - Review and Editing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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