Research Paper

Effects of *Cyathula officinalis* Kuan Extracts on Hypertension-induced Renal Vascular Remodelling by Inhibiting the Expression of ALD, Renin, Ang II and the Activation of Erk1/2 and P38 Signalling Pathways: A Mechanistic Study

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The aim of this study was to investigate the role of the extracts of *Cyathula officinalis* Kuan on blood pressure in spontaneously hypertensive rats and the possible mechanisms involved. Blood pressure was recorded, renal vascular remodelling was visualized through haematoxylin and eosin staining and the expression of aldosterone, renin and angiotensin II in serum and kidney of spontaneously hypertensive rats was measured by real-time polymer chain reaction and enzyme-linked immuno-sorbent assay, respectively. The extracts of *Cyathula officinalis* Kuan significantly decreased the high blood pressure and reduced renal artery narrowing in spontaneously hypertensive rats. Moreover, *Cyathula officinalis* Kuan extracts also decreased the expression of aldosterone, renin and angiotensin II in both the serum and the kidney and activated ERK1/2 and p38 signalling pathways in kidney of spontaneously hypertensive rats. However, the toxicity to rat liver and kidney did not differ significantly between the extracts of *Cyathula officinalis* Kuan and enalapril, a well-known prodrug providing antihypertensive actions. These results demonstrated that the extracts from *Cyathula officinalis* Kuan can ameliorate hypertension-induced renal vascular remodelling in a rat models through inhibiting the expression of aldosterone, renin and angiotensin II and activating ERK1/2 and p38 signalling pathways.

Key words: *Cyathula officinalis* Kuan, spontaneously hypertensive rats, renal vascular remodelling, renin-angiotensin-aldosterone system

Clinical hypertension is classified as essential hypertension and secondary hypertension. Essential hypertension is an independent disease that defined high blood pressure as the main clinical manifestation due to nonspecific lifestyle and genetic factors[1,2], accounting for more than 90 % of all hypertensive patients, and its pathogenesis remains unknown. Hypertension is one of the most common cardiovascular disease, characterized by high incidence, high mortality and high disability, and the incidence of the disease in adults was 18.8 % according to the 2002 epidemiology research of China[3]. High blood pressure can affect the structure and function of important organs such as heart, brain and kidney. Various complications caused by hypertension seriously threaten the life and quality of life of patients[4,5]. Prevention and treatment of hypertension is thus not only a problem of lowering blood pressure, but also of slowing or preventing damage to target organs. It is a hot issue in the world to find a new target to protect the target organ and to prevent the complications of hypertension[6]. Currently, it is generally believed that vascular remodelling is a

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structural and functional change of blood vessels in the process of growth, development, aging and diseases[7]. In the context of hypertension, vascular remodelling allows arteries to withstand the increased pressure load, but as a result, the vessels typically become more rigid than in their native state, and the reduced compliance decreases their ability to dampen the cyclical changes in blood pressure[8]. Vascular remodelling is not only an important pathophysiological change and the basis of pathogenesis of some cardiovascular diseases such as hypertension and diabetes, but also one of the important pathogenic mechanisms that cause damage to target organs[9,10]. Changes in blood pressure, which can lead to changes in wall thickness, are the first factors considered to be associated with vascular diameter and function[11]. Hypertension can cause vascular remodelling, and vascular remodelling can also cause increased vascular resistance. The vicious cycle of the two can eventually cause damage to target organs and functional insufficiency[12].

Extensive systemic artery involvement is the direct cause of pathological changes in target organs in hypertension. Small artery lesions are the most important pathological changes in hypertension. The small artery lesions in the tissues and organs can promote the maintenance and development of hypertension, and eventually lead to the ischemic injury of tissues and organs[13], among which renal arterial lesions are the most obvious injuries[14], and these verity of lesions is closely related to the blood pressure and the duration of the disease[15]. Persistent hypertension results in the vascular wall thickening, lumen stenosis, glomerular fibrosis, atrophy and renal arteriosclerosis, which ultimately lead to the renal failure[16]. The root of *Cyathula officinalis* Kuan is a widely used medicinal herb in China with a wide range of pharmacological activities[17]. The main components of *C. officinalis* extracts are alkaloids, including cyasterone, inokosterone and ecdysterone. The spontaneously hypertensive rat (SHR) is a well-characterized model representing primary hypertension of humans, which has obvious structural and functional changes in the early stage of hypertension. In larger renal cortical artery of 4-w old SHR, the area of the vessel middle wall and ratio of wall to cavity were increased and obviously with age accompanied by the damage and dysfunction of target organ[18,19]. *C. officinalis* extracts could decrease blood pressure and influence myocardial cell diameter in SHR, and the mechanism of antihypertensive effect might be associated with the inhibition of angiotensin converting enzyme and Ang II production, which leads to vasodilation and a reduction of blood pressure[9]. However, it is still unknown how *C. officinalis* extracts inhibit high blood pressure via its influences on vascular remodelling in the kidneys of SHR. To elucidate the effects of *C. officinalis* extracts on blood pressure and renal vascular remodelling in SHR, the expression of aldosterone (ALD), renin and angiotensin II (Ang II) and the activation of ERK1/2 and p38 signaling pathways were measured. In this investigation, *C. officinalis* extracts significantly ameliorated hypertension-induced renal vascular remodeling through inhibiting the expression of ALD, renin and Ang II and the activation of ERK1/2 and p38 signaling pathways.

MATERIALS AND METHODS

Experimental animals:

Forty four male SHR rats (12 w, 256±20 g) were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China), and housed in polypropylene cages with sawdust bedding in hygienically controlled environment (23-25°) with a 12-h light/dark cycle throughout the study period. All animal care and procedures were in strict accordance with the China Laboratory Animal Use Regulations and were approved by the Institutional Animal Care and Use Committee at Putuo District people's Hospital of Shanghai City (Shanghai, China).

Extraction procedures:

The dried *C. officinalis* plant material (1.6 kg) was powdered and refluxed with 85 % ethanol for 10 times (3 times×1.5 h). The extracts were concentrated under vacuum and dried, and the so obtained *C. officinalis* extract yield was 79.2 g. *C. officinalis* extract (47 g) was resuspended in distilled water and submitted to sequential extraction with petroleum ether, dichloromethane, and n-butanol (5 times), to obtain the n-butanol fraction, which was concentrated under vacuum and dried, obtaining the ACO (33.5 g). The samples were stored at -4° until used.

Animal treatments:

Animals were randomly divided into 5 groups, SHR control group (n=8); 2.5 mg/kg enalapril-treated SHR group (Shanghai Shyndec Pharmaceutical Co., Ltd, Shanghai, China; n=8); 3 g/kg *C. officinalis* extracts (Shaanxi Jiahe Phytochem Co., Ltd, Xian, China; n=9); 6 g/kg *C. officinalis* extracts (n=9); 12 g/kg

C. officinalis extracts (n=10). Animals in the treatment group were administered intragastrically with 2.5 mg/kg enalapril, 3, 6 or 12 g/kg *C. officinalis* extracts once a day for 8 w. Blood pressure was measured once a week in the conscious state using a tail BP Series Automatic non-invasive blood pressure measuring system (BP-300A; Chengdu Techman Software Co., Ltd, Chengdu, China). Prior to the measurement of blood pressure, rats were trained to get accustomed to the equipment. Animals were anesthetized using intraperitoneal injection of 30 mg/kg pelltobarbitalum natricum (pentobarbital sodium) after 8 w treatment. The kidney was harvested and placed in 4 % paraformaldehyde for haematoxylin and eosin staining.

RNA extraction and real-time reverse transcription polymerase chain reaction:

Total RNA from the kidney was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and 2 µg of RNA was reverse transcribed with PrimeScript RT reagents Kit according to the manufacturer's instructions. Real-time PCR was carried out using SYBR Green (Takara, Otsu, Shiga, Japan) and performed using the GeneAmp PCR Systems 2700 (Applied Biosystems). The primer sequences for polymerase chain reaction (PCR) were as follows; ALD forward, 5'-CTCAGCACCAAAGCACAAATC-3'; ALD reverse, 5'-AGTAGGCACAACCCAGTAATC-3'; renin forward, 5'-CGGCATAACAATCGCATC-3'; renin reverse, 5'-AAGGGACAAGCACTCATC-3'; GAPDH forward, 5'-GTCGGTGTGAACGGATTTG-3'; GAPDH reverse, 5'-TCCCATTCTCAGCCTTGAC-3'. Expression values were calculated using the comparative Ct method and the GAPDH gene was used as endogenous control.

Western blot:

The whole cell extracts from the kidney were prepared using RIPA buffer (Beyotime, Shanghai, China). After electrophoresis, proteins were electroeluted at 120 volts onto a polyvinylidene difluoride membrane. The membrane was incubated with primary antibodies against antiALD (Life Span BioSciences, Inc, LS-C27137), antirenin (Abcam, ab176127), antiphospho-ERK1/2 (CST,#9101), antiERK1/2 (Abcam, ab17942), antiphospho-p38 (CST,#9211), antip38 (CST,#9212), and antiGAPDH (CST, #5174) overnight at 4° and then incubated with a secondary antibody. GAPDH was used as a control for total protein input.

Enzyme-linked immunosorbent assay (ELISA):

Secretions of ALD, renin and Ang II were determined by ELISA. Briefly, plasma collected from SHR with different treatments were mixed with 15 µl 10 % EDTA and 20 µl 200 IU aprotinin and then centrifuged at 400×g at 4° for 10 min. The serum was harvested and stored at -20°. The relative content of ALD, renin and Ang II in the serum of SHR was measured using an ELISA kit according to the manufacturer's protocol.

Measurement of the toxicity of *C. officinalis* extracts:

The content of plasma alanine aminotransferase (ALT) and creatinine (CRE) was measured by Beckman Coulter Chemistry analyser AU5800 Series (Beckman Coulter Commercial Enterprise (China) Co. Ltd). The content of plasma C-reactive protein (CRP) and brain natriuretic peptide (BNP) was measured using ELISA as previously described.

Statistical analysis:

Results were shown as mean±SD. All data were analyzed by SPSS 18.0 software (SPSS, Inc., Chicago, USA). Comparison was done with one-way ANOVA followed by post hoc test. *P* value of less than 0.05 was considered statistically significant.

Ethical consideration:

All *in vivo* experiments were performed in accordance with the regulations for the administration of experimental animals in our university. The animal protocols were approved by the Animal Care and Use Committee (IACUC) of our University under reference No.7643/MMU/CH.

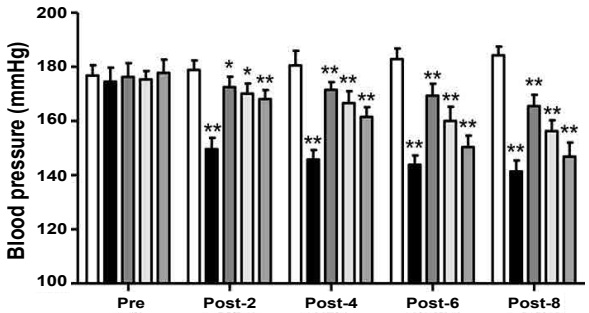

Fig. 1: Effect of enalapril and *Cyathula officinalis* extracts on blood pressure in SHR

The extracts of *Cyathula officinalis* decreased the blood pressure in SHR. The blood pressure in SHR before or after treatment with 2.5 mg/kg enalapril or *Cyathula officinalis* extracts (3, 6 or 12 g/kg) for 2, 4, 6 and 8 weeks was measured. **p<0.01 compared with SHR. (□) SHR; (■) SHR+enalapril; (■) SHR+3 g/kg; (■) SHR+6 g/kg; (■) SHR+12 g/kg

RESULTS AND DISCUSSION

In the SHR with 2.5 mg/kg enalapril treatment for 2, 4, 6 and 8 w, the blood pressure was measured and found to be significantly decreased by 16.4, 19.2, 21.3 and 23.3 % compared to the SHR before enalapril treatment. SHR treated with different doses of *C. officinalis* extracts (3, 6 and 12 g/kg) treatment for 2, 4, 6 and 8 w, exhibited a significant fall in the blood pressure compared to SHR before enalapril treatment, with the most reduction in blood pressure was detected in the 12 g/kg *C. officinalis* extracts-treated group (fig. 1). In the SHR treated with 12 g/kg *C. officinalis* extracts for 2, 4, 6 and 8 weeks, the blood pressure was significantly decreased by 6.1, 10.5, 17.8 and 23.3 % compared to that of the SHR before treatment, respectively (fig. 1).

The structure of kidney and renal arteries was examined on histological sections. As shown in fig. 2A, SHR with different doses of *C. officinalis* extracts significantly prevented the glomerular fibrosis and sclerosis as well as renal atherosclerosis compared with the SHR without treatment, with the most effective prevention detected in 6 g/kg *C. officinalis* extracts-treated group, which had a similar effect as 2.5 mg/kg enalapril in SHR. Furthermore, SHR with different doses of *C. officinalis* extracts also significantly reduced the thickness of the vascular wall and inhibited the decreased retinal arterial diameter compared with the SHR without treatment, with the most effective prevention detected in 6 g/kg *C. officinalis* extracts group, which had a similar effect as 2.5 mg/kg enalapril in SHR (fig. 2B).

Abnormal activity of the renin-angiotensin-aldosterone system (RAAS) leads to the development of hypertension, atherosclerosis, myocardial infarction, stroke, congestive heart failure and renal disease[20,21]. Therefore, the expression of ALD, renin and Ang II in serum and kidney of SHR with different treatment was measured by real-time PCR and ELISA, respectively. The concentration of serum ALD, renin and Ang II of SHR with 2.5 mg/kg enalapril or different doses of *C. officinalis* extracts (3, 6 and 12 g/kg) treatment was significantly decreased compared to that in SHR without treatment (fig. 3A). As shown in (fig. 3B), the content of renal renin in 2.5 mg/kg enalapril or different doses of *C. officinalis* extracts (3, 6 and 12 g/kg) group showed significant decrease compared with that in SHR without treatment. The mRNA and protein expression of ALD and renin in kidney of SHR with 2.5 mg/kg enalapril or different doses of *C. officinalis* extracts (3, 6 and 12 g/kg) treatment was significantly decreased compared with that in SHR without treatment (fig. 3C-E).

Given the role that ERK1/2/p38 pathways as down stream effectors of RAAS mediated vascular biology and physiology, particularly, vascular remodeling[21], the expression of ERK1/2 as well as p38 and their phosphorylation levels was also examined by western blotting. As shown in (figs. 3D and F), the expression of p-ERK1/2and p-p38 in kidney of SHR with 2.5 mg/kg enalapril or different doses of *C. officinalis* extracts (3, 6 and 12 g/kg) treatment significantly decreased compared to that in SHR without treatment. However, the expression of ERK1/2 and p38 was not changed after treatment. These results suggested that *C. officinalis* extracts inhibited the activation of ERK1/2 and p38 signaling in SHR.

To investigate the toxicity of *C. officinalis* extracts on liver and kidney function, the content of ALT, CRE,

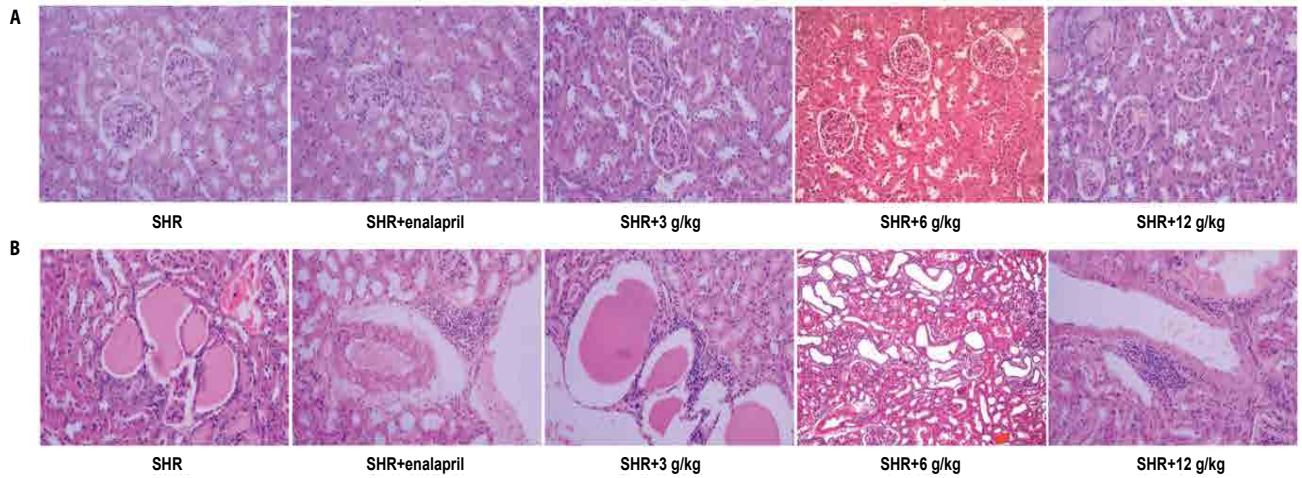

Fig. 2: Effects of enalapril and *Cyathula officinalis* extracts on renal vascular remodeling in SHR
***Cyathula officinalis* extracts inhibited renal vascular remodeling in SHR. Morphological characteristics of renal artery in SHR treated with 2.5 mg/kg enalapril or *Cyathula officinalis* extracts (3, 6 or 12 g/kg) for 8 w. Magnification of A. ×40 and B. ×100**

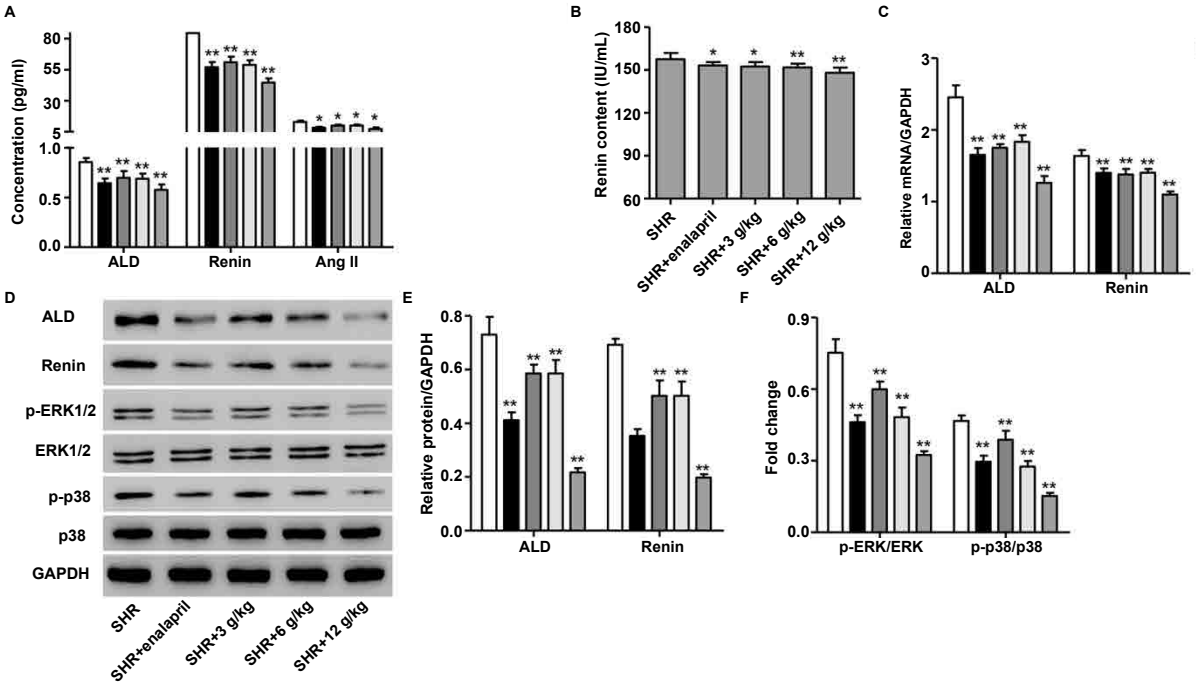

Fig. 3: Effects of enalapril and *Cyathula officinalis* extracts on renin, ALD and Ang II in both serum and kidney of SHR
SHR treated with 2.5 mg/kg enalapril or extracts of *Cyathula officinalis* (3, 6 or 12 g/kg) for 8 w. A. Concentrations of ALD, renin and ang II in serum measured by ELISA, B. content of renin in kidney of SHR measured by ELISA, C. mRNA and protein expression of ALD, renin, p-ERK1/2, ERK12, p-p38, and p38 in kidney was measured by real-time PCR and D-F. Western blot. **P<0.01 compared with SHR. (□) SHR; (■) SHR+enalapril; (■) SHR+3 g/kg; (■) SHR+6 g/kg; (■) SHR+12 g/kgR

TABLE 1: EFFECT OF *CYATHULA OFFICINALIS* KUAN EXTRACTS ON THE CONTENT OF ALT, CRE, CRP AND BNP

Group	ALT (IU/ml)	Cre (μM)	CRP (ng/ml)	BNP (ng/ml)
SHR	30.76±6.87	38.60±3.14	74.06±3.49	53.46±1.85
SHR+12 g/kg	28.56±6.17	48.56±4.75**	51.23±3.96**	41.53±4.20**
SHR+6 g/kg	26.68±5.50	47.98±3.09**	52.25±4.93**	40.92±0.91**
SHR+3 g/kg	26.91±6.46	48.40±3.51**	51.07±2.41**	42.63±3.58**
SHR+enalapril	28.52±7.93	48.01±2.76**	49.17±3.79**	40.17±1.46**

SHR- spontaneously hypertensive rat, ALT- alanine aminotransferase, CRE- creatinine, CRP- plasma C-reactive protein and BNP- brain natriuretic peptide. **P<0.01 compared with SHR

CRP and BNP was measured in plasma of SHR. As shown in (Table 1), enalapril or different doses of *C. officinalis* extracts significantly increased the content of CRE, decreased the content of CRP and BNP and had no effect on ALT content in SHR compared to the SHR without treatment. However, there were no significant differences on the content of ALT, CRE, CRP and BNP between enalapril and different doses of *C. officinalis* extracts.

To the best of our knowledge, the present study is the first to determine the effects of *C. officinalis* extracts on renal vascular changes in hypertensive animals. The main results and findings of this work are as follows, firstly, *C. officinalis* extracts decrease blood pressure in SHR; secondly, these extracts prevented the glomerular fibrosis and sclerosis as well as renal atherosclerosis in SHR; thirdly, these extracts reduced the thickness of the vascular wall and inhibited the decreased retinal arterial diameter in SHR; fourthly, and most importantly, *C. officinalis* extracts inhibited the expression of ALD, renin and Ang II in both serum and kidney of SHR.

Hypertension is associated with decreased renal function and renal failure. The kidney is an important organ to regulate the balance of water and electrolyte and has a variety of endocrine functions. It is not only an important organ of blood pressure regulation, but also one of the main target organs of hypertension[22,23]. It is well known that resistance vessels become thicker or encroach into the lumen in kidneys[24], which occurs mainly in the preglomerular vessels at the prehypertensive or early stage of hypertension and that promotes hypertension by increasing renal vascular resistance and reducing both glomerular filtration and

sodium excretion in SHR[25]. Hypertension increases peripheral vascular resistance by vascular remodelling, including vascular smooth muscle cell proliferation, hypertrophy, vascular compliance and narrow vessel lumen. In previous studies, it was also reported that vascular remodelling ultimately leads to target organ damage, such as myocardial hypertrophy, myocardial fibrosis and glomerular hyalinization in SHR[26]. Recent study have reported that *C. officinalis* extracts significantly reduced the expression of renal TGF-1 in SHR through inhibiting renal fibrosis, renin release and Ang II production, thus leading to vasodilation and a decrease in blood pressure[27]. In the present study, the protective effect of *C. officinalis* extracts against retinal vascular remodeling was confirmed, indicating the potential protection against end-organ damage induced by hypertension, such as renal damage.

The RAAS, one of the most important endocrine systems, is responsible for the regulation of blood pressure, fluid volume, sodium and potassium balance in cardiovascular, renal and adrenal glands[20]. It plays an important role in the occurrence and development of hypertension, leads to the development of cardiovascular and renal diseases and contributes to the drug resistance[21]. In the present study, *C. officinalis* extracts were found to significantly decrease the expression of ALD, renin and Ang II in both serum and kidney of SHR. The primary function of renin is to eventually cause an increase in blood pressure, leading to restoration of perfusion pressure in the kidneys. Renin inhibition is indeed associated with lowering of Ang II levels and blood pressure reduction[28]. Ang II, the most active substance of RAAS, has a strong role in vascular remodeling involving vasoconstriction and promotion of development of atherosclerosis, endothelial dysfunction, hypertension and related diseases such as metabolic syndrome, through a variety of inflammation and coagulation mechanism[29,30]. Another effector of the RAAS, ALD, plays a central role in the regulation of plasma Na^+, extracellular K^+ and arterial blood pressure[31].

ERK1/2 and p38 MAPK signalling pathways play important roles in cell proliferation and extracellular matrix deposition during hypertensive cardiovascular remodeling[21,32]. In the present study, *C. officinalis* extracts significantly inhibited the activation of ERK1/2 and p38 signalling pathways. ALD mediates vascular biology and physiology, particularly, vascular remodelling, fibrosis and vascular tone by regulating ERK1/2 and p38 signalling pathways[21]. p38 MAPK inhibition improves vascular remodeling and vascular function in Ang II-induced hypertension[33]. Therefore, it can be postulated that *C. officinalis* extracts might inhibit hypertension-induced renal vascular remodeling in rat models through inhibiting the expression of ALD, renin and Ang II and downstream effectors ERK1/2 and p38 signaling pathways.

In order to further evaluate the potential of this drug in clinic, the toxicity of *C. officinalis* extracts on human liver and kidney was studied by measuring the plasma level of ALT, CRE, CRP and BNP in SHR. The results clearly showed that the *C. officinalis* extracts significantly decreased ALT, CRP and BNP plasma level and increased CRE content in plasma in SHR. However, compared to enalapril, a well-known antihypertensive, *C. officinalis* extracts did not affect the plasma level of these indexes, suggesting little toxicity of *C. officinalis* extracts to human liver and kidney.

In conclusion, this work demonstrated for the first time that *C. officinalis* extracts ameliorated hypertension-induced renal vascular remodelling in the SHR model through inhibiting the expression of ALD, renin and Ang II and activation of ERK1/2 and p38 signalling pathways. It suggested that *C. officinalis* extracts have potential beneficial effects on the renal vascular remodelling induced by hypertension.

Conflict of interest:

No conflict of interest between any of the authors.

Acknowledgements:

This work was funded by the Research Project of Shanghai Municipal Health and Family Planning Commission (20144Y0134), Key projects in Yangpu District (YP16ZC04) and Construction Project of Key Clinical Specialty of Traditional Chinese Medicine in Putuo District (PTZYLCZDZK-2017006).

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This article was originally published in a special issue, "XXXXXX"

Indian J Pharm Sci 2020:82(1)spl issue1;1-7