Original Research

Venthamarai chooranam, a polyherbal Siddha medicine, alleviates hypertension via AT_1R and *eNOS* signaling pathway in 2K1C hypertensive rats

Chidambaram S Babu[1], Periyathambi Kalaivani[1], Vijayan Ranju[1], Sekar Sathiya[1], Chandrasekar Anbarasi[2], Murugappapillai V Mahadevan[1], Harivanzan Vijayakumar[2], Adoor G Sunil[1] and Sadagopan Thanikachalam[1,2]

[1]Centre for Toxicology and Developmental Research (CEFT), Sri Ramachandra University, Chennai 600 116, Tamil Nadu, India; [2]PURSE-HIS Study, Sri Ramachandra University, Chennai 600 116, Tamil Nadu, India
Corresponding author: S Thanikachalam. Email: ceftpublications@gmail.com

Abstract

The present study was aimed to scientifically demonstrate the anti-hypertensive action of Venthamarai chooranam (VMC) in renal hypertensive rats. Two Kidney One Clip (2K1C) Goldblatt model was adopted to induce hypertension in rats. Male Sprague Dawley rats (270–320 g) were randomized into sham ($n = 6$), vehicle-treated 2K1C ($n = 9$) and VMC-treated 2K1C (400 mg/kg, p.o; $n = 8$) and monitored for nine weeks. Systolic blood pressure (SBP), plasma nitrate/nitrite, carotid endothelial nitric oxide synthetase (eNOS), renal angiotensin type 1 receptor (AT_1R), angiotensin type 2 receptor (AT_2R), TNFα, IL-6, thioredoxin 1 (TRX1), and thioredoxin reductase 1 (TRXR1) mRNA expressions were studied. VMC upregulated eNOS expression which in turn improved plasma nitric oxide and decreased SBP in hypertensive rats. It down-regulated AT_1R and simultaneously upregulated AT_2R expression in comparison to vehicle-treated 2K1C rats. Further, renal TNFα and IL-6 expressions were down-regulated while TRX1 and TRXR1 were upregulated by VMC. VMC potentially interacts with renin-angiotensin components and endothelial functions, and thereby exerts its antihypertensive action. This is the first study to demonstrate the mechanism of anti-hypertensive action of VMC in an animal model of renovascular hypertension.

Keywords: Renovascular hypertension, Siddha system, 2K1C, Venthamarai chooranam, AT_1R, AT_2R, eNOS

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Introduction

Angiotensin II (Ang II) mediates its physiological actions primarily through angiotensin type 1 receptors (AT_1Rs).[1,2] Over-activation of AT_1Rs leads to the development of Ang II-dependent hypertension, production of pro-inflammatory cytokines[3,4] and oxidative stress.[5] Stimulation of angiotensin type 2 receptors (AT_2R) plays counter-regulatory effect in the control of blood pressure [6] and inflammation-mediated vascular injuries.[7] Decreased nitric oxide (NO) production by deregulated endothelial NO synthetase (eNOS) is implicated in the pathogenesis of hypertension. Blockade of AT_1Rs or stimulation of AT_2Rs was shown to increase NO production.[8] AT_1R blockers and angiotensin converting enzyme inhibitors (ACEIs) are effective candidates in the management of hypertension and associated pathological degenerations that occur due to altered renin angiotensin system (RAS) functions. But, the most common side-effects of ACEIs include dry cough, hyperkalemia, low blood pressure, and dizziness.[9] On the other hand, AT_1R blockers are well tolerated and non-productive cough is much less when compared to ACEIs. Serious side-effects such as angioneurotic edema, liver damage, renal failure, aggravation of angina, and migraine are also rarely reported with AT_1R blockers.[9,10]

Traditional medicines are gaining significant attention in global health debates because of the cost-effectiveness and safety associated with their long-term administration. Siddha system of medicine is one of the most ancient traditional medical systems existing since the days of Dravidian culture in Tamil Nadu, India. In many instances, the traditional diagnostic and treatment modalities of Siddha system have been claimed to be highly beneficial. Though many of the concepts and advantages of Siddha system remain untested by modern day science, herbal-based Siddha medicines seem to be widely preferred for various chronic ailments in the present days.

Venthamarai chooranam (VMC), a poly-herbal Siddha medicine, has been used for the treatment of hypertension for more than three decades in Tamil Nadu. Clinical records of Siddha practitioners indicate that VMC produces significant anti-hypertensive effect with no or mild side-effects in secondary hypertensive patients. It contains crude powders of *Nelumbo nucifera*, *Elaettaria cardamomum*, *Zingiber officinale*, *Piper longum*, *Glycyrrhiza glabra*, *Anethum graveolens* and *Cuminum cyminum* (composition shown in Table 1).

Two kidney One Clip (2K1C) renovascular hypertension is a renin-angiotensin system dependent experimental model. Development of inflammation and generation of free radicals in the clipped kidneys, followed by progressive renal fibrosis in 2K1C model mimics the clinical pathophysiological features of chronic renal failure and secondary hypertension.[11,12] To the best of our knowledge, we have not found any scientific report on the anti-hypertensive mechanism of VMC. Hence, the present study was undertaken to demonstrate the effect of VMC in one of the secondary hypertension models namely renovascular hypertension in rats.

Materials and methods

Chemicals and reagents

Carboxy methyl cellulose (CMC), sulphanilamide, and N-(1-naphthyl) ethylenediamine dihydrochloride were purchased from SISCO Research Laboratories, Mumbai, India. 5-Sulpho salicylic and ortho-phosphoric acid was supplied by Himedia laboratories, Mumbai, India. All other chemicals, reagents, and solvents used were of analytical grade.

Venthamarai Chooranam

VMC was procured commercially from Arogya Healthcare Pvt Ltd, Chennai, Tamil Nadu, India and stored according to the manufacturer's recommendation (at room temperature). It was subjected to preliminary phytochemical analysis and was estimated for tannins, flavonoids, and total phenols content.

Standardization of VMC by HPTLC

Standardization was performed to ensure that there is no batch to batch variability (qualitative and quantitative) in the chemical constituents of VMC. Since VMC is a poly-herbal formulation containing seven herbs, we standardized for quercetin content, by HPTLC, a chemical marker that is already reported for anti-hypertensive effect and herein it is present in a majority of VMC's individual ingredients.[13]

Table 1 Composition of Venthamarai Chooranam (VMC)

S. No.	Plants	Composition (g)
1	*Nelumbo nucifera*	105
2	*Elaettaria cardamomum*	5
3	*Zingiber officinale*	10
4	*Piper longum*	5
5	*Glycyrrhiza glabra*	20
6	*Anethum graveolens*	25
7	*Cuminum cyminum*	30

Animals, Husbandry and Ethics approval

Adult male and female Sprague Dawley rats were procured from Central Animal Facility, Sri Ramachandra University, Chennai. Animals were housed in groups (3–5 animals/polypropylene cage) under an ambient temperature of $22 \pm 3^{\circ}C$ and 40–65% relative humidity, with a 12-h light/dark artificial photoperiod in a well-ventilated room (air cycle: 15 changes/hr; recycle ratio: 70:30). They were provided with rodent feed (Provimi Animal Nutrition India Pvt. Ltd, Bangalore) and purified water ad libitum. Animals were acclimatized for seven days to laboratory condition prior to the initiation of experiment. Guidelines of 'Guide for the Care and Use of Laboratory Animals' (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number #85-23, revised 1996) were strictly followed throughout the study. Institutional Animal Ethics Committee (IAEC), Sri Ramachandra University, Chennai, India approved the study protocol (IAEC-XIII/SRU/88/2008).

Acute oral toxicity of VMC

Acute toxicity of VMC was performed according to the OECD test guideline 423 for testing of chemicals in fasted female Sprague Dawley rats (120–130 g). LD_{50} cut-off value of VMC was determined in accordance with the Globally Harmonized System (GHS) of Classification and Labelling of chemicals.[14]

2K1C surgical procedure

Male Sprague Dawley rats weighing 270–320 g were used for the study. Blood pressure was measured in all the animals once every three days for three weeks so as to adapt them to tail-cuff plethysmography (model MC4000; Hatteras Instruments, North Carolina) before the surgical procedure. The values thus obtained were used as baseline SBP. 2K1C surgical procedure was performed as described earlier[15] but with slight modifications. Animals were anesthetized with ketamine (75 mg/kg i.p) and Midazolam (1 mg/kg i.p). Under aseptic conditions, left kidney was exposed through a small flank incision, externalized and handled carefully with an ophthalmic chalazion forceps. For clipping, the renal artery of the left kidney was individualized over a short segment by blunt dissection and a silver clip (0.22 mm i.d.) was placed close to the renal artery, resulting in partial occlusion of renal perfusion. The kidney was then gently pushed back into the retroperitoneal cavity and wound was closed with a running 3-0 silk suture. In sham-operated rats, the surgical procedure was implicated without artery clipping. Animals were placed in individual cages and enrofloxin (5 mg/kg i.p) was administered upon recovery as antibiotic.

Post-operative care was performed for the animals as per the IAEC guidelines.

Two weeks after the complete healing of surgical wound, SBP was measured for one week. Animals with SBP $\geq$ 160 mmHg were selected and randomized to three groups, sham ($n = 6$) and 2K1C received vehicle for 63 days (10 ml/kg CMC [0.5%], p.o; $n = 9$) and 2K1C received VMC for 63 days (400 mg/kg, p.o; $n = 8$).

Measurement of body weight and SBP

Body weights were recorded weekly and dose of VMC was adjusted accordingly. SBP was measured once in every three days for 63 days.

Determination of mRNA expression

Gene expression of carotid eNOS and renal AT_1R and AT_2R, TNFα and IL-6, TRX1 and TRXR1 was performed by reverse transcriptase-PCR as described earlier.[16] Primers sequence used were as follows. AT_1R: sense, 5′-CTT TCT TCT CAA TCT CGC CTT GG-3′; antisense, 5′-CCA GAA AGC CGT AGA ACA GAG GG-3′. AT_2R: sense, 5′-CCT TCT TGG ATG CTC TGA CC-3′; antisense, 5′-TGG AGC CAA GTA ATG GGA AC-3′. eNOS: sense, 5′-CAC CCT CAG GTT CTG TGT GTT-3′; antisense, 5′-GTA GCC TGG AAC ATC TTC CGT-3′. TNFα: sense, 5′-CCA CGT CGT AGC AAA CCA CCA AG-3′; antisense, 5′-CAG GTA CAT GGG CTC ATA CC-3′. IL6: sense, 5′-GAG GAT ACC ACC CAC ACC AGA CCA GTA-3′; antisense, 5′-GGT TTG CCG AGT AGA CCT CAT AG TGA C-3′. TRX1: sense, 5′-GCC AAA ATG GTG AAG CTG A-3′; antisense, 5′-CTG GCA GTC ATC CAC GTC T-3′. TRXR1: sense, 5′-GTA GAT TAG CCA AGT CAC CG-3′; antisense, 5′-GTC AGA TGG CTT AGT AGA GC-3′and β-actin: sense, 5′-TGC TGT CCC TGT ATG CCT CT-3′; antisense, 5′-AGG TCT TTA CGG ATG TCA ACG-3′.

Plasma NO measurement

Plasma NO was measured in terms of nitrate/nitrite content as described earlier.[17] Briefly, nitrate/nitrite (NO_2/NO_3) was assayed by taking 0.2 ml of plasma followed by addition of 1.8 ml of saline and 0.4 ml of 35% sulphosalicylic acid for protein precipitation. The precipitate was removed by centrifugation at 4000 rpm for 10 min. To 1 ml aliquot of supernatant, 2 ml Griess reagent (1 g of sulphanilamide dissolved in small volume of water, 2 ml of orthophosphoric acid, and 100 mg of naphthyl ethyldiamine were added. Volume was made up to 100 ml with distilled water and mixed well). The mixture was allowed to stand for 20 min under dark conditions. The colour intensity was read at 540 nm (Perkin Elmer, λ25, USA). Standard calibration was plotted using sodium nitrite in the concentration range 200–1000 ng.

Heart, common carotid artery, and renal histopathology

After 63 days of treatment, experimental animals were euthanized under carbon-di-oxide. Heart, common carotid arteries, and renal tissues obtained from the experimental animals were fixed in 10% neutral buffered formalin, dehydrated, and paraffin embedded. Then, 5 μm sections were cut and stained with hematoxylin and eosin. In addition, heart sections from the experimental animals were subjected to Masson's trichrome staining for detection of collagen. Sections were examined by veterinary pathologist who was blinded to treatment protocol under light microscope (Motic DMB1–2MP, China).

Statistical analysis

Data were expressed as mean ± standard error mean (mean ± SEM). Mean difference in body weight change in acute toxicity, SBP, kidney weights between groups were analyzed by unpaired Student 't' test. Biochemical and molecular data were analyzed by one way ANOVA followed by Tukey's multiple comparison as *post hoc* test. $P \leq 0.05$ was considered to be statistically significant. Statistical analysis was performed using GraphPad Prism 5.0, San Diego, CA, USA.

Results

Standardization of VMC

Preliminary phytochemical analysis of VMC revealed the presence of tannins, flavones, poly phenols, proteins, glycosides, reducing sugars, anthroquinones, quinones, alkaloids, and saponins. Total phenolic, tannins, and flavonoid contents of VMC were found to be 23.45 ± 1.36, 5.67 ± 2.36, and 43.33 ± 3.46 % w/w, respectively.

Quercetin quantification by HPTLC

Quercetin content was analyzed in three different batches of VMC and it was found to be 0.19 ± 0.06 % w/w (Figure 1).

Acute oral toxicity in rats

VMC did not produce mortality or any signs of toxicity in the experimental animals at 2000 mg/kg, p.o. There was no significant difference in body weight between vehicle and VMC-treated animals (Table 2). Gross pathological examination revealed no abnormalities. Thus, in reference to the GHS Classification and Labelling of chemicals, VMC can be classified as Category 5 and concluded to be a safer medicine.

Body and kidney weights

There was no significant difference in body weights between the groups (Table 3). Nine weeks after renal artery clipping, vehicle-treated 2K1C rats showed significant ($P<0.01$) decrease in clipped kidneys weight (atrophy) and significant ($P<0.01$) increase in non-clipped kidneys weight (hypertrophy) relative to sham-operated rats. VMC did not prevent either of the changes and, in fact, increased the weights of non-clipped kidneys (Table 4)

Systolic blood pressure

No significant difference in systolic blood pressure (SBP) was observed in the experimental animals before the surgery. Two weeks after 2K1C procedure, SBP was found to be significantly ($P<0.01$) increased in vehicle-treated rats than in sham-operated rats. Further, the vehicle-treated 2K1C

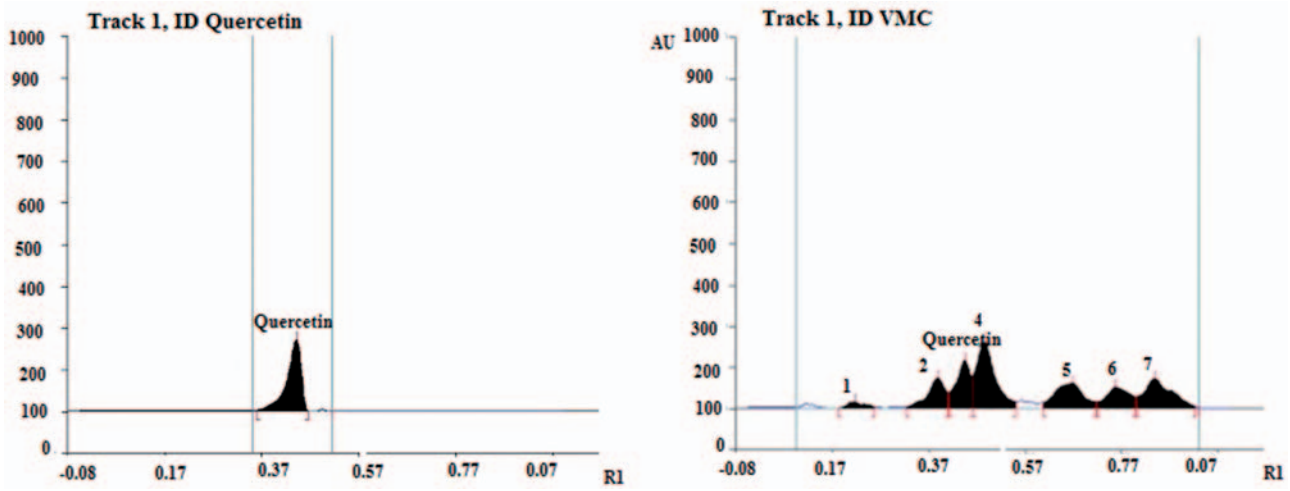

Figure 1 Standardization of VMC by HPTLC. (A color version of this figure is available in the online journal)

Table 2 Effect of Venthamarai Choornam (VMC) on body weight in acute oral toxicity study

	Body weight (g)					
Treatment	-1 day	-1 h before VMC administration on day 1	6 h after VMC administration on day 1	Day 2	Day 7	Day 14
0.5% CMC	141.33 ± 0.88	131.33 ± 0.88	134.00 ± 0.58	139.67 ± 0.33	144.00 ± 1.15	150.00 ± 0.58
VMC	139.00 ± 1.53	128.67 ± 0.88	132.00 ± 2.31	137.00 ± 2.65	143.67 ± 1.45	147.67 ± 0.88

Note: Values are expressed in mean ± SEM; $n = 3$ per group.

rats showed persistent SBP elevation in the post-surgery weeks. Treatment with VMC for a period of 63 days significantly decreased SBP when compared to the vehicle-treated 2K1C rats (Figure 2).

eNOS expression in common carotid arteries and plasma NO levels

eNOS expression was found to be down-regulated ($P<0.05$) in vehicle-treated 2K1C rats when compared to sham rats. VMC significantly ($P<0.05$) upregulated eNOS expression in 2K1C rats (Figure 3).

In comparison to sham rats, the plasma NO level was found to be significantly ($P<0.01$) decreased in vehicle-treated 2K1C rats. VMC significantly ($P<0.01$) increased NO content when compared to vehicle-treated 2K1C rats (Figure 4).

Renal expression of AT_1R and AT_2R

Renal AT_1R ($P<0.05$) and AT_2R ($P<0.01$) expressions were significantly upregulated in the clipped kidneys of vehicle-treated 2K1C rats when compared to sham rats. VMC significantly ($P<0.01$) down-regulated AT_1R expression in clipped kidneys. Interestingly, VMC increased AT_2R mRNA expression additively in clipped kidney of 2K1C rats. A significant ($P<0.05$) increase in AT_1R with no change in AT_2R mRNA expressions was observed in the non-clipped kidneys of 2K1C rats when compared to the sham-operated rats (Figure 5(a) and (b)).

Inflammatory markers

In comparison to sham-operated rats, TNFα ($P<0.05$) and IL-6 ($P<0.01$) expressions were upregulated in clipped kidneys of vehicle-treated 2K1C rats. VMC significantly down-regulated TNFα ($P<0.05$) and IL-6 ($P<0.01$) expression in clipped kidneys. Non-significant increase in TNFα and IL-6 mRNA expressions were observed in non-clipped kidneys of 2K1C rats when compared to sham rats (Figure 5(c) and (d)).

Oxidative stress markers

TRX1 and TRXR1 mRNA expressions were down-regulated ($P<0.05$) in the clipped kidney of vehicle-treated rats when compared to sham operated rats. VMC treatment reversed these alterations significantly ($P<0.05$) in the clipped kidneys. There were no differences in TRX1 and TRXR1 expressions in non-clipped kidneys between the experimental groups (Figure 5(e) and (f)).

Heart, common carotid arteries and renal histopathology

Sham-operated rats revealed normal architecture of cardiac muscles and blood vessels. Mild degree of myocardial

Table 3 Effect of Venthamarai Choornam (VMC) on body weight changes in renal hypertension

Treatment	Body weight (g)								
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63
Sham operated	332.33 ± 2.67	349.17 ± 6.73	353.67 ± 6.84	349.83 ± 6.73	356.83 ± 6.21	343.33 ± 14.71	351.50 ± 5.59	356.83 ± 5.99	359.33 ± 1.36	370.17 ± 9.73
2K1C + Vehicle (0.5% CMC)	332.33 ± 9.15	328.67 ± 10.49	338.00 ± 10.59	338.00 ± 11.10	346.33 ± 11.20	341.83 ± 15.14	348.33 ± 11.79	355.00 ± 12.30	344.00 ± 12.72	355.50 ± 11.94
2K1C + VMC (400 mg/kg, p.o)	337.17 ± 8.86	338.50 ± 8.15	332.83 ± 6.70	320.00 ± 6.77	331.17 ± 6.09	320.67 ± 7.99	328.50 ± 4.16	327.17 ± 10.10	320.00 ± 6.77	339.33 ± 6.68

Note: Values are expressed in mean ± SEM; $n = 6$–9 per group.

Table 4 Effect of Venthamarai choornam (VMC) on clipped and non-clipped kidneys' weight in two kidneys/one clip (2K1C) hypertensive rats

Treatment	Kidney weight (g)	
	Clipped kidneys	Non-clipped kidneys
Sham operated	1.28 ± 0.04	1.30 ± 0.02
2K1C + Vehicle (0.5%CMC)	0.84 ± 0.03$^{\#\#}$	1.55 ± 0.05$^{\#\#**++}$
2K1C + VMC (400 mg/kg, p.o)	0.78 ± 0.09$^{\#\#}$	1.90 ± 0.09$^{\#\#**++}$

Note: Values are expressed in mean ± SEM; $n = 6$–9 per group.
$^{\#\#}P < 0.01$ vs sham operated.
$^{**}P < 0.01$ vs respective clipped kidneys.
$^{++}P < 0.01$ vs clipped 2K1C group.

degeneration with inflammatory foci and focal area of perivascular mononuclear cells infiltration were observed in vehicle-treated hypertensive animals. VMC-treated group revealed mild degree of perivascular mononuclear cells infiltration and inflammatory foci in the myocardium (Figure 6(a) to (c)). There were no differences in collagen intensities around the blood vessels and in myocardium between the experimental groups. Further, no fibrosis was observed in the heart sections, in particular the left ventricle of the hypertensive rats (Figure 7(a) to (c)).

Histopathological examination of common carotid artery and kidneys of sham-operated rats revealed normal histological pattern. Carotid arteries of vehicle-treated 2K1C rats displayed increased medial wall thickness due to smooth muscle cell hyperplasia and hypertrophy while VMC rats revealed normal carotid artery histology similar to those of sham-operated rats (Figure 8).

Clipped kidneys of vehicle and VMC-treated rats showed multifocal to generalized tubular degeneration with marked increase in interstitial mononuclear cells infiltration. Non-clipped kidneys of vehicle treated 2K1C rats revealed diffused renal hypertrophy with multifocal hypertrophic arteriolar wall, progressive narrowing of the lumina and hyalinosis, while the renal histology remained almost normal in the non-clipped kidneys of VMC-treated rats; however, there was mild mesangial proliferation of small cortical arteries comparable to sham-operated rats (Figure 9).

Discussion

The present study shows that VMC exerts its anti-hypertensive effect through its interactions with RAS components and by ameliorating inflammation and oxidative stress.

eNOS is the predominant source for the production of NO in vasculature and thus plays a crucial role in the regulation of blood pressure and vascular tone.[11] VMC increased eNOS mRNA expression and plasma NO production in 2K1C rats. This increased NO might diffuse into the vascular smooth muscles in the vicinity to promote vasodilation. This effect could be the probable reason for the observed decrease in SBP in 2K1C rats, which closely resembles ARBs action.[10,18]

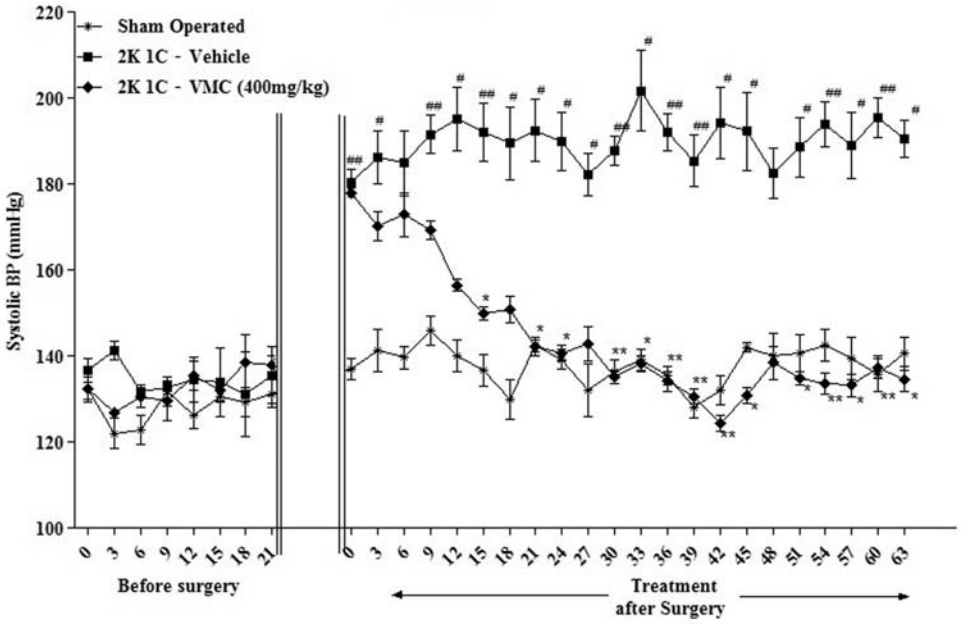

Figure 2 Effect of Venthamarai Chooranam (VMC) on systolic blood pressure in 2K1C rats

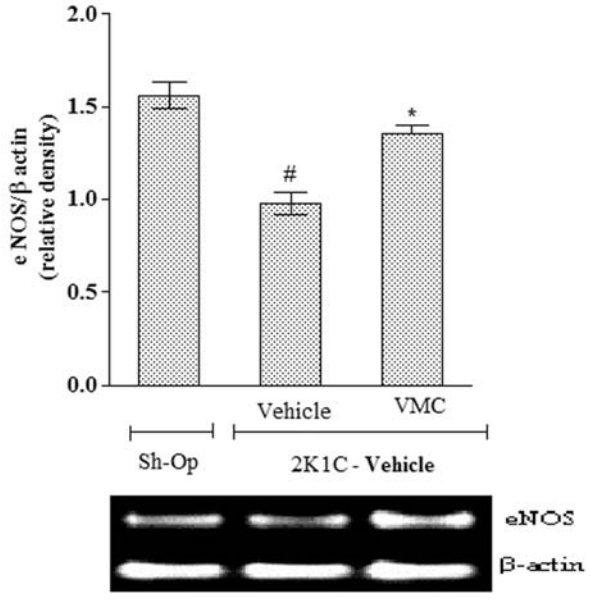

Figure 3 Carotid endothelial expression of endothelial nitric oxide synthetase (eNOS) in sham, 2K1C + vehicle and 2K1C + Venthamarai Chooranam (VMC) rats

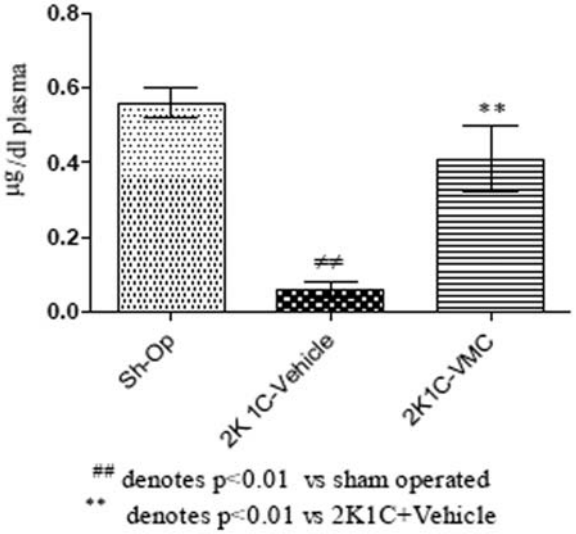

Figure 4 Effect of Venthamarai Chooranam (VMC) on plasma Nitric oxide (NO) level in 2K1C rats

NO inhibits vascular growth by inactivating platelet-derived growth factor.[19] Histopathological examination of carotid arteries of vehicle-treated 2K1C rats revealed smooth muscle cells proliferation and hypertrophy. Decreased eNOS in turn the NO levels could be the causative factors for the observed vascular smooth muscle cells proliferation and hypertrophy in hypertensive rats. VMC treatment reversed these vascular changes which clearly demonstrate its recuperative effect on eNOS and NO levels.

Alterations in angiotensin receptors expression play a crucial role in the pathogenesis of renal vascular hypertension. AT_1R blockade is reported to increase AT_2R gene promoter activity.[20] On the other hand, over-expression of AT_2R was shown to down-regulate AT_1R expression in rat vascular smooth-muscle cells.[21] VMC down-regulated AT_1R and increased AT_2R levels in 2K1C rats which reveals that the restoration of blood pressure is through its potential interactions with the renin-angiotensin system.

VMC did not prevent the atrophy of the clipped kidneys, rather exacerbated the hypertrophy of non-clipped kidneys. But, the role of AT_1R and AT_2R in the change of weight in kidneys is unclear.

In the current study, increased TNFα and IL-6 mRNA expression in 2K1C rats were found to be associated with

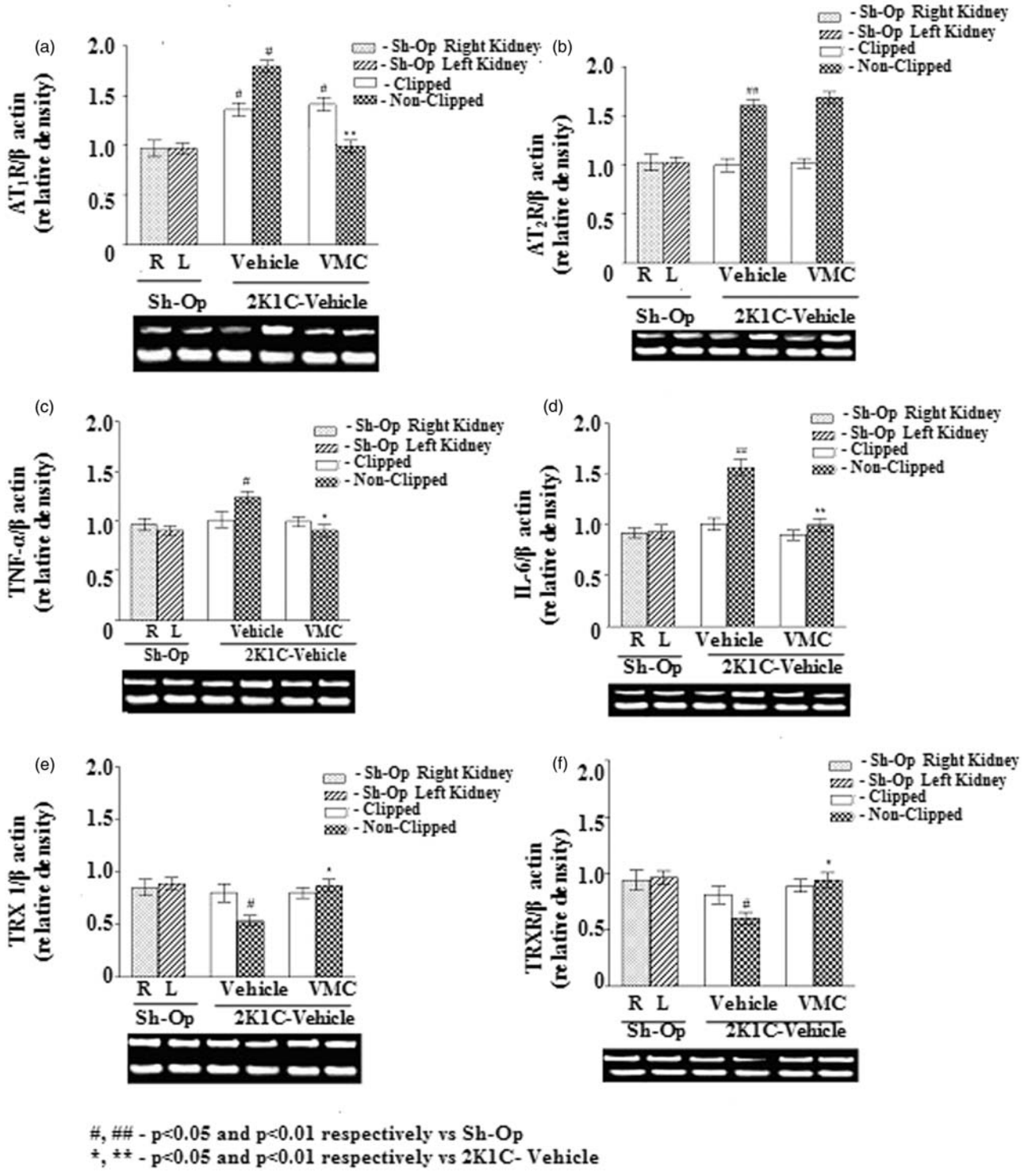

Figure 5 Renal mRNA expression of Angiotensin Type 1 Receptor (AT_1R) (a), Angiotensin Type 2 Receptor (AT_2R) (b), Tumor Necrosis Factor Alpha TNFα (c), Interleukin-6 (IL-6) (d), Thioredoxin 1 (TRX1) TRX1 (e), and Thioredoxin Reductase 1 (TRXR1) (f) in right (R) and left (L) kidneys of Sham; non-clipped (open bars) and clipped (dark dotted bars) kidneys of vehicle and Venthamarai Chooranam (VMC)-treated 2K1C rats. Bottom: Representative mRNA bands. Data were expressed in mean ± SEM

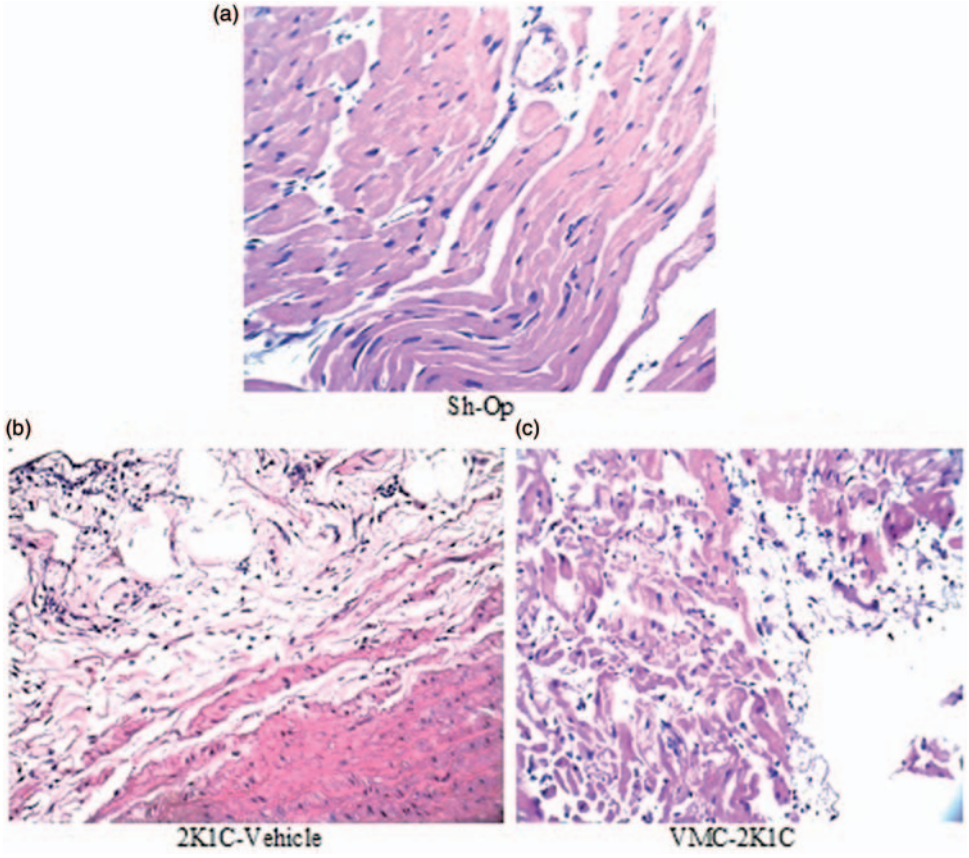

Figure 6 Hematoxylin eosin staining of heart in Sham-operated (a), 2K1C + vehicle (b), and 2K1C + Venthamarai Chooranam (VMC) (c) rats. (A color version of this figure is available in the online journal.)

the infiltration of inflammatory cells in clipped kidneys and these events could be a consequence of AT_1R over-expression. Pharmacological blockade of AT_1R is reported to reduce renal inflammation and enhance NO production[3] through AT_2R.[22] Further, stimulation of AT_2R is also reported to produce NO[8] and inhibit renal renin synthesis.[23] VMC suppressed TNFα and IL-6 expression in clipped kidneys without any effect on the non-clipped kidneys. This differential effect of VMC might be due to the difference in density of RAS components such as angiotensin II, renin, AT_1R/AT_2R between clipped and non-clipped kidneys. Increased eNOS and NO levels with AT_1R and AT_2R mRNA expressions in 2K1C rats strongly suggest that VMC potentially interacts with angiotensin II receptors. However, at this juncture it is not possible to characterize whether VMC is an AT_1R blocker or AT_2R activator.

Oxidative stress roots to eNOS dysfunction such that it no longer produces NO, but reactive oxygen species ($O_2^{\bullet}$).[24,25] TRX1 and TRXR1 are unique antioxidant systems that interact with various intracellular signaling pathways and are key regulators of several transcriptional factors including NFκ-B.[26] NFκ-B is involved in the regulation of TNFα and IL-6 transcription in primary Substance P stimulated mast cells.[27] Decreased plasma NO with arterial eNOS and renal TRX1 along with TRXR1 mRNA down-regulation reflects the prevalence of severe oxidative stress in the 2K1C rats. Further, this decreased TRX1 and TRXR1 would have activated NFκ-B and hence resulted in the increase of TNFα and IL-6 expression. This observation was also found to be consistent with an earlier report.[28] ACE inhibitors and AT_1R antagonists are reported to possess anti-inflammatory and antioxidant actions.[29,30] Increased TRX1 and TRXR1 and decreased TNFα and IL-6 levels demonstrate the ameliorative effect of VMC against oxidative stress and inflammation in the vascular structures of 2K1C rats. Histopathological examination showed no fibrosis in left ventricles or alterations in collagen bed intensity around the myocardial blood vessels in vehicle or VMC-treated 2K1C rats which might be due to the shorter experimental schedule.

Flavonoids constitute a large group of secondary metabolites in plants and are richly present in dietary sources such as apples, onions, grapes, berries, tea, etc. Large body of evidences support that the dietary intake of flavonoids can exert beneficial effects on vascular structure and functions and also reduce the risk for cardiovascular morbidity and mortality.[31–34] Lorenz *et al*. demonstrated that flavonoids have the ability to activate eNOS via PI3-K, PKA, and Akt pathway.[35] Further, they are reported to trigger a specific pattern of eNOS phosphorylation that is associated with increased NO synthesis.[36,37] Siddha medicine VMC has been used for the treatment of hypertension for

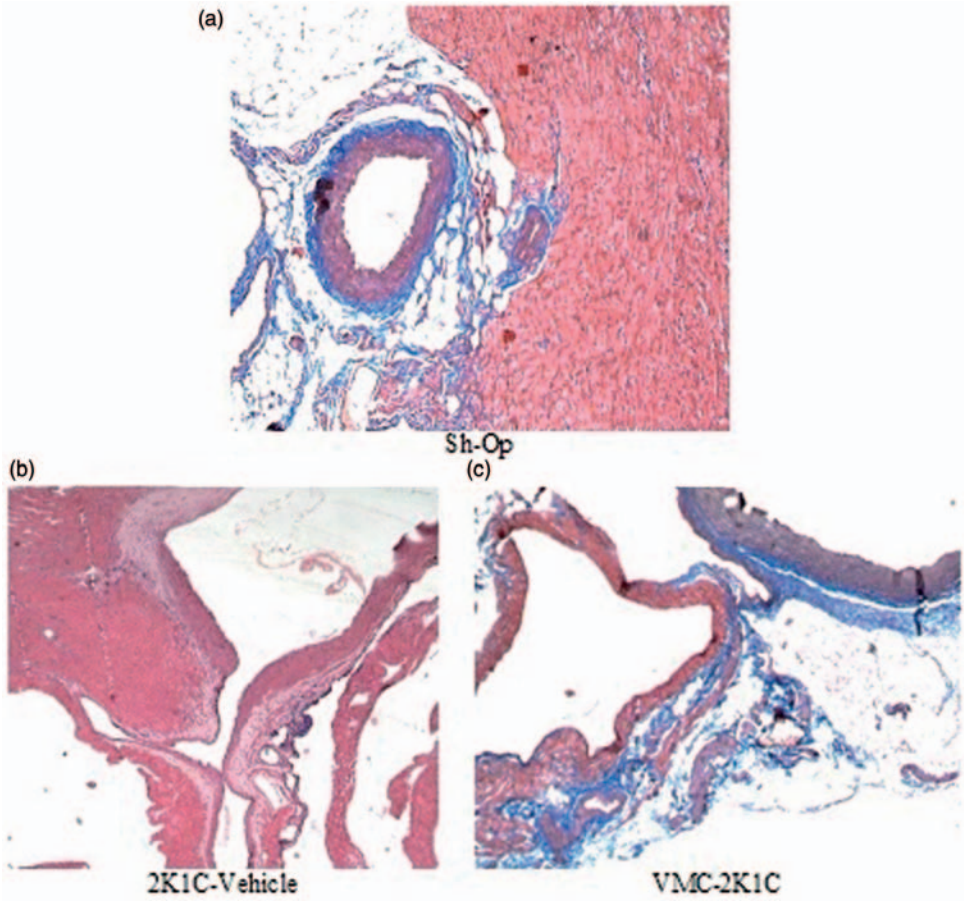

Figure 7 Masson's tricrome staining of heart in Sham-operated (a), 2K1C + vehicle (b), and 2K1C + Venthamarai Chooranam (VMC) (c) rats. (A color version of this figure is available in the online journal.)

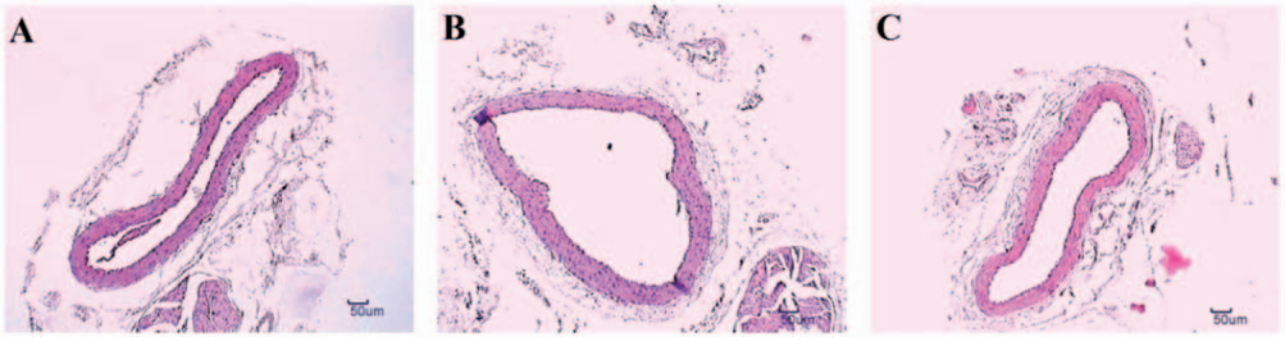

Figure 8 Representative photographs of hematoxylin and eosin stained coronary artery of Sham (a); 2K1C + vehicle (b), and 2K1C + Venthamarai Chooranam (VMC) (c). (A color version of this figure is available in the online journal.)

more than three decades without any alterations in its formula. The interesting fact about VMC is that the constituent herbs of this preparation are easily available and routinely used in food ingredients in India, especially in South India. Analysis of secondary metabolites revealed that VMC contains higher concentration of flavonoids (43.33 ± 3.46 %w/w). In addition, HPTLC standardization shows reasonable concentration of quercetin in VMC, which has earlier been reported to lower blood pressure and improve endothelial function.[38] This finding brings up the conjecture that flavonoids might be one of the active principles in VMC, at least in part. Correlating the physiological and molecular datum, it also becomes evident that VMC possesses significant anti-hypertensive effect, which was found to be exerted through the modulation of eNOS/NO pathway. Current study also suggests that VMC interacts with renin-angiotensin system and in turn counteracts oxidative stress and vascular inflammation (Figure 10).

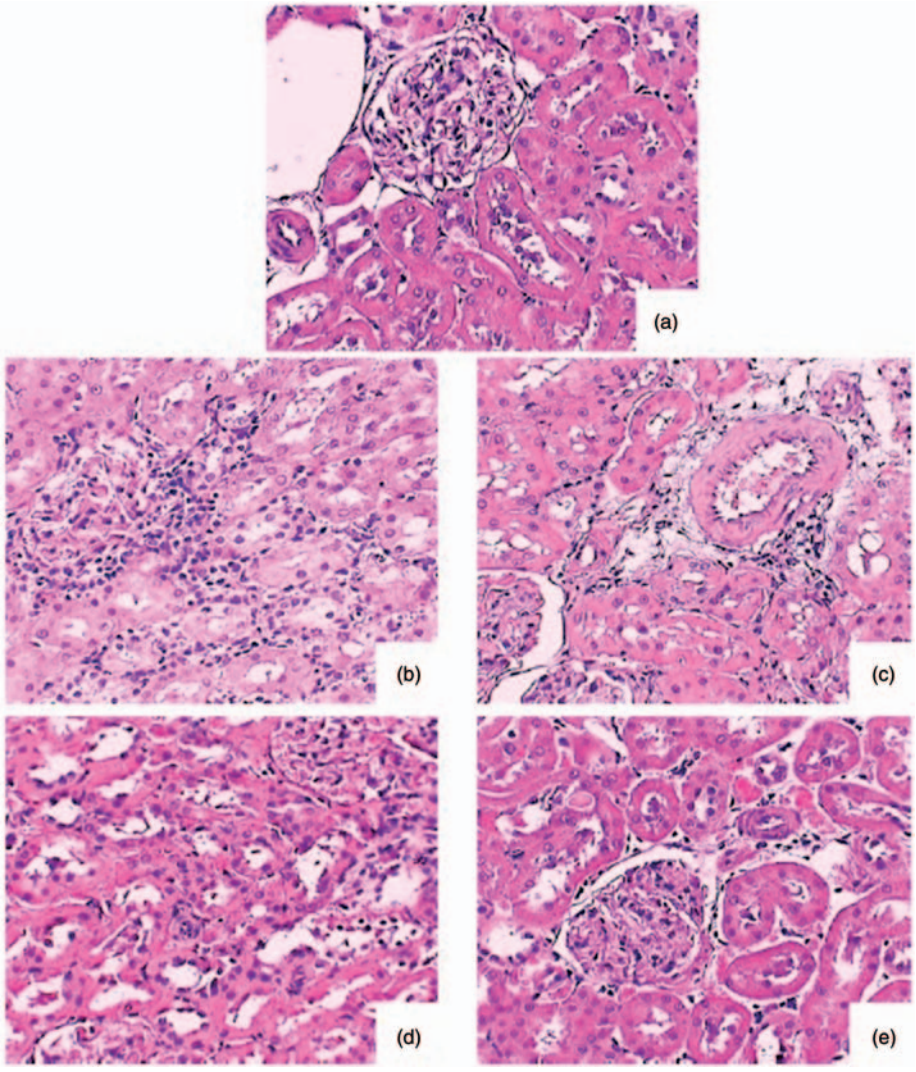

Figure 9 Representative photographs of hematoxylin and eosin stained kidney of Sham (a); 2K1C + vehicle clipped (b) and non-clipped (c); and 2K1C + VMC clipped (d) and non-clipped (E). (A color version of this figure is available in the online journal.)

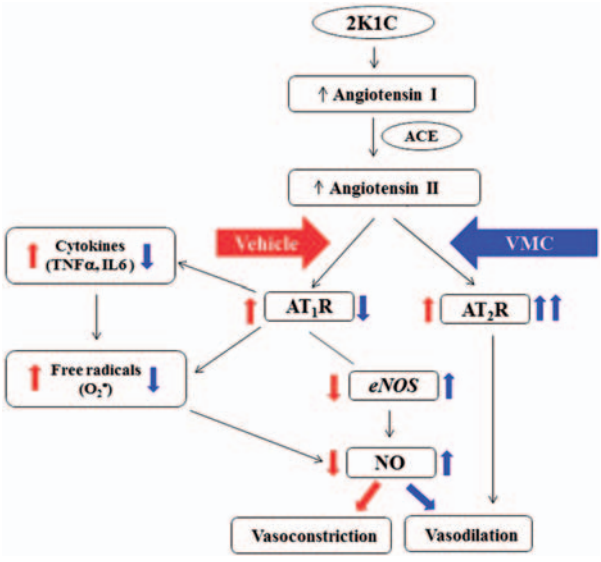

Figure 10 Schematic representation of interaction of VMC with renin-angiotensin system. (A color version of this figure is available in the online journal.)

Conclusions

In summary, this study evidences that Venthamarai chooranam can upregulate arterial eNOS and plasma NO levels in renal hypertensive state. It has the ability to down-regulate the over expressed AT_1Rs and augment AT_2Rs expression. In addition, VMC can potentially suppress the pro-inflammatory cytokines and oxidative stressors. Chemical standardization of VMC revealed substantial amount of flavonoids, in particular quercetin, and this emphasizes that flavonoids might be the active principle, at least in part, for the observed antihypertensive effect.

Author Contributions: CSB performed the study, interpreted the data and drafted the manuscript; PK performed the study; SS performed the study and statistical analysis; VR assisted in the performance of the study and manuscript preparation; CA, MVM, HV and AGS assisted in the performance of the study; ST designed and organized the study and work plan, supervised the study, interpreted the data and corrected and finalized the manuscript.

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