**Cuminum cyminum**, a Dietary Spice, Attenuates Hypertension via Endothelial Nitric Oxide Synthase and NO Pathway in Renovascular Hypertensive Rats

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Abstract

**Cuminum cyminum** (CC) is a commonly used spice in South Indian foods. It has been traditionally used for the treatment and management of sleep disorders, indigestion, and hypertension. The present study was carried out to scientifically evaluate the anti-hypertensive potential of standardized aqueous extract of CC seeds and its role in arterial endothelial nitric oxide synthase expression, inflammation, and oxidative stress in renal hypertensive rats. Renal hypertension was induced by the two-kidney one-clip (2K/1C) method in rats. Systolic blood pressure (SBP), plasma nitrate/nitrite, carotid endothelial nitric oxide synthase expression, inflammation, and oxidative stress in renal hypertensive rats; Renal hypertension was induced by the two-kidney one-clip (2K/1C) method in rats. Systolic blood pressure (SBP), plasma nitrate/nitrite, carotid endothelial nitric oxide synthase expression, inflammation, and oxidative stress in renal hypertensive rats. It also up-regulated the gene expression of eNOS, Bcl-2, TRX1, and TRXR1; and down-regulated Bax, TNF-α, and IL-6. These data reveal that CC seeds augment endothelial functions and ameliorate inflammatory and oxidative stress in hypertensive rats. The present report is the first of its kind to demonstrate the mechanism of anti-hypertensive action of CC seeds in an animal model of renovascular hypertension.

Keywords: hypertension, 2K/1C, *Cuminum cyminum*, endothelial function, inflammation, oxidative stress

INTRODUCTION

Hypertension is an important public health challenge worldwide. It is a major risk factor for stroke, myocardial infarction, heart failure, aneurysms of arteries, and peripheral arterial diseases, and is a causative factor in chronic kidney disease. Endothelial nitric oxide synthase (eNOS) plays a significant role in hypertension and associated complications. Decreased bioavailability of eNOS-derived nitric oxide (NO) and reduction in nitric oxide synthase (NOS) levels were responsible for the impairment of endothelial functions in hypertension (1). The impairment in nitric oxide synthesis triggers oxidative stress through generation of peroxynitrite radicals using superoxides (2). Diuretics, β-blockers, calcium channel blockers (CCBs), angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs) etc. are widely used anti-hypertensive agents in the treatment of primary and secondary hypertension (3). While considering the clinical benefits of these anti-hypertensive agents, it is inevitable to put forth their side effects, which may vary with individual drugs/classes. The most common side effects of anti-hypertensive agents include hypotension, fatigue; a few more effects include cough, itchy skin rash, dizziness, severe vomiting, diarrhea, fast or irregular pulse, chest pain, angioneurotic edema, swelling of the face and tongue, hyperkalemia etc. (4,http://www.bupa.co.uk/individuals/healthinformation/directory/a/ace-inhibitors; http://www.medicinenet.com/angiotensin_i_receptor_blockers/article.htm#side) Drugs that modulate the renin angiotensin system, such as ACE inhibitors, produce cough and their prolonged use affects kidneys function while ARBs have no reports of serious side effects (http://www.bupa.co.uk/individuals/healthinformation/directory/a/ace-inhibitors; http://www.
Since triterpenoids were reported to be one of the major active principles in CC, it was standardized for triterpenoids content (18).

**Experimental Animals, Husbandry, and Ethical Approval**

Male Sprague Dawley rats (320–340 g) were obtained from Central Animal Facility, Sri Ramachandra University, Chennai. Rats were fed with rodent extruded pellet feed (Provimi, India) with purified water ad libitum. They were housed in a well-controlled environment of 22 ± 3°C temperature under artificial photoperiod of 12-h light/12-h dark cycle. Experimental animals were acclimatized for 7 days to the laboratory conditions prior to the start of the study. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC), Sri Ramachandra University, Chennai (IAEC/XXII/SRU/165/2011).

**Investigation Protocol**

Renal hypertension was induced in the rats by the two-kidney/one-clip (2K1C) method with slight modifications (19). Prior to surgical procedure, rats were adapted to the systolic blood pressure (SBP) measurement procedure; BP was measured once in 3 days for 3 weeks by tail-cuff plethysmography (model MC 4000; Hatteras Instruments, Cary, NC, USA). Post-operative care was given to the rats as per the institutional guidelines. Two weeks following post-operative care, SBP was measured for 1 week. Animals with SBP ≥ 160 mm Hg were selected and randomized to two groups: the 2K1C group served as positive control and received the vehicle (0.5% CMC, p.o.; n = 6) and the 2K1C-CC group served as test group, which received CC (200 mg/kg, p.o.; n = 6) for 63 days. Sham-operated rats received vehicle and served as control. Dose of CC was selected based on earlier studies (20,21). SBP was measured once in 3 days for 63 days and body weight was recorded weekly. At the end of the treatment schedule, experimental animals were euthanized under excessive isoflurane to collect kidneys and carotid artery. Weights of clipped and non-clipped kidneys were recorded and the organs were processed for further evaluation.

**mRNA Expression**

mRNA expression of carotid–eNOS and renal–TNF-α, IL-6, Bax, Bcl-2, TRX1, and TRXR1 was performed as described earlier (22), by RT-PCR technique and semi-quantified using Bio1D software in gel documentation (Vilber Lourmat, France). The primer sequence used was as follows. TNF-α: sense, 5'-CCA CGT CGT AGC AAA CCA CGA AG-3'; antisense, 5'-CAG GTA CAT GGG CTC ATA CC-3' IL-6: sense, 5'-GAG GAT ACC ACC CAC ACC AGA CCA GTA-3'; antisense, 5'-GTT TGG CCG AGT AGG CAG TGA C-3'. Bax: sense, 5'-GAG TGT CTC CGG CGA ATT G-3'; antisense, 5'-TGG TGA GGG AGG CCG TGA G-3'. Bcl-2: sense, 5'-GCG GAG ATC GTG ATG AAG T-3'; antisense, 5'-CCA CGG AAC TCA AAG AAG G-3'. eNOS: sense, 5'-CAC CCT CAG GTT CTG TGT GTT-3';
antisense, 5′-GTA GCC TGG AAC ATC TTC CGT-3′; β-actin: sense, 5′-TGC TGT CCC TGT ATG CCT CT-3′; antisense, 5′- AGG TCT TTA CGG ATG TCA ACG -3′, TRX1: sense 5′ TTC CTT GAA GTA GAC GTG GAT GAC 5′; antisense -5′AGA GAA CTC CCC AAC CTT TTG AC3′; antisense; 5′ATT TGT TGC CTT AAT CCT GTG AGG-3′.

Plasma Nitric Oxide
Nitric oxide release was measured in terms of nitrate/nitrite levels following Griess et al methods (23). To 0.2 mL of plasma, 1.8 mL of saline and 0.4 mL of 5-sulfosalicylic acid (35%) were added to precipitate protein. The precipitate was removed by centrifugation at 4000 rpm for 10 minutes. To 1 mL of supernatant, 2 mL Griess reagent was added and mixed well. The mixture was allowed to stand for 20 minutes in dark and the color intensity was read at 540 nm in spectrophotometer (Thermo Scientific Multiskan, Scan Jose, CA, USA).

Histopathology
Common carotid artery and kidneys were fixed in 10% neutral buffered formalin, dehydrated, and paraffin embedded following the standard protocol. Sections were cut to 5 μm thickness, stained with hematoxylin and eosin, and examined under light microscopy.

Statistical Analysis
Data were expressed as mean ± standard error of mean (mean ± SEM). Mean difference between groups was determined by one-way ANOVA followed by Tukey multiple comparison as a post hoc test. P value ≤ .05 was considered significant. Statistical analysis was performed using Graph Pad prism 5.0 (San Diego, CA, USA).

RESULTS
Phytochemical Analysis
Qualitative analysis of aqueous extract of CC seeds revealed high phenols, flavones, tannins, and alkaloids content. Total phenolic, tannins, flavonoids, and triterpenoids contents of CC were found to be 35.80 ± 0.73%, 10.62 ± 0.83%, 16.98 ± 1.82%, and 22.12 ± 2.33% w/w, respectively.

Effect of *Cuminum cyminum* on Body Weight and Kidneys Weight
Throughout the treatment period, no significant difference in body weight was observed between experimental groups (Figure 1). Relative weights of clipped and non-clipped kidneys of experimental animals are presented in Table 1. Vehicle-treated 2K/1C rats showed significant (P < .01) decrease in clipped kidney weight and increase in non-clipped kidney weight after 9 weeks of renal artery clipping when compared to sham-operated rats. Treatment with CC did not alleviate the weight loss in clipped kidneys.

*Cuminum cyminum* Decreased Systolic Blood Pressure (SBP)
There was no significant difference in baseline SBP value between the experimental animals. Two weeks after surgery, SBP was significantly (P < .01) elevated in vehicle-treated 2K/1C rats when compared to sham-operated rats. Vehicle-treated 2K/1C rats showed persistent elevation in SBP during post-surgery weeks (9 weeks). On the other hand, treatment with CC significantly reduced SBP in 2K/1C rats when compared to vehicle-treated rats (Figure 2).

![Figure 1. Effect of CC on body weight in 2K/1C rats.](image-url)
Cuminum cyminum Improved eNOS Expression in Common Carotid Arteries

eNOS expression was found to be down-regulated ($P < .05$) in vehicle-treated 2K/1C rats when compared to sham rats. *Cuminum cyminum* treatment up-regulated eNOS expression significantly ($P < .05$) when compared to vehicle-treated 2K/1C rats (Figure 3).

*Cuminum cyminum* Suppressed Inflammatory Markers (TNF-α and IL-6) Expression

mRNA expression of TNF-α and IL-6 in clipped and non-clipped kidneys of 2K/1C rats are shown in Figure 4A and 4B. TNF-α and IL-6 were significantly up-regulated in the clipped kidneys of 2K/1C group when compared to sham-operated group. Treatment with CC significantly ($P < .05$) down-regulated these cytokines levels in clipped kidneys when compared to vehicle-treated 2K/1C rats. On the other hand, IL-6 expression was up-regulated in non-clipped kidney of vehicle-treated 2K/1C rat; nevertheless, the observation was non-significant when compared to sham-operated group. However, CC treatment ameliorated these changes in non-clipped kidney.

Table 1. Effect of *C. cyminum* on relative kidney weight of 2K/1C operated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Clipped</th>
<th>Non-clipped</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operated</td>
<td>0.34 ± 0.01</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>2K/1C + vehicle (0.5% CMC)</td>
<td>0.18 ± 0.03*</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>2K/1C + <em>C. cyminum</em> (200 mg/kg, p.o.)</td>
<td>0.22 ± 0.08</td>
<td>0.43 ± 0.02</td>
</tr>
</tbody>
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Values are expressed as mean ± SEM; $n = 6$/group; significance with Tukey test following one-way ANOVA is indicated as $^*P < .01$ versus Sham operated.
**Cuminum cyminum** Down-Regulated Bax and Up-Regulated Bcl-2 Levels

Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) expressions were studied in both clipped and non-clipped kidneys of experimental rats (Figure 5A and 5B). Significant ($P < .01$) up-regulation in Bax and down-regulation in Bcl-2 expression were observed in clipped kidneys of vehicle-treated 2K/1C rats when compared to the sham-operated rats. *Cuminum cyminum* significantly ($P < .01$) reversed these alterations when compared to vehicle-treated 2K/1C rats. In non-clipped kidneys, Bax expression was up-regulated in both vehicle-treated and CC-treated 2K/1C rats; nonetheless, the observations were non-significant.

**Cuminum cyminum** Attenuated Oxidative Stress

TRX1 and TRXR1 expressions were significantly ($P < .05$) down-regulated in the clipped kidneys of vehicle-treated 2K/1C rats compared to sham-operated rats (Figure 5C and 5D). Treatment with CC significantly ($P < .05$) up-regulated their expression in comparison to vehicle-treated 2K/1C rats. However, no significant alteration in TRX1 and TRXR1 expression was observed in non-clipped kidney.

**Plasma NO (Nitrate/Nitrite)**

Plasma NO content was significantly ($P < .01$) reduced in vehicle-treated 2K/1C rats. *Cuminum cyminum* significantly ($P < .01$) increased plasma NO content when compared to vehicle-treated 2K/1C rats (Figure 5E).

**Histopathology**

Histopathological examination of common carotid artery of the sham-operated group revealed a normal histological pattern (Figure 6A–C). Carotid artery of vehicle-treated 2K/1C rats showed increased thickening of arterial wall because of smooth muscle cell hyperplasia and hypertrophy with increased collagen content. *Cuminum cyminum* rats showed normal carotid artery histology similar to sham-operated rats. Sham-operated rats revealed normal renal histological pattern (Figure 7A–E). Clipped kidneys of vehicle-treated 2K/1C rats revealed multifocal to generalized tubular degeneration accompanied by interstitial mononuclear cell infiltrations. *Cuminum cyminum* treatment significantly reduced the infiltration of inflammatory cells in the renal cortex region of clipped kidneys. Non-clipped kidneys of the vehicle-treated 2K/1C rats showed diffuse renal hypertrophy with multifocal hypertrophic arteriolar wall, and progressive narrowing of the lumina and hyalinosis. Whilst the renal histology remained normal in the non-clipped kidneys of CC and vehicle-treated 2K/1C rats, mild mesangial proliferation of small cortical arteries was observed and it was comparable to that of the sham-operated rats.

**DISCUSSION**

Renovascular hypertension induced by 2K/1C surgical method is a renin and nitric oxide pathway dependent animal model. It is a complex phenomenon, defined by secondary elevation of blood pressure, which interferes...
with renal arterial circulation and causes ischemia. Balance between NO, inflammatory markers, and vascular generation of reactive oxygen species is crucial for maintaining cardiovascular and renovascular homeostasis, particularly in the regulation of vascular tone and remodeling (24).

Endothelial dysfunction and decreased production of nitric oxide by deregulated eNOS are implicated in...
the pathogenesis of hypertension (25,26). Endothelial-derived NO is one of the major vasorelaxant autacoids, which in addition to the systemic control of blood pressure also exerts paracrinic control of microvascular tone in kidneys (27). In the present study, CC treatment improved eNOS mRNA expression and plasma nitric oxide production in 2K/1C rats. This increased NO might have diffused into the vascular smooth muscle cells and would have promoted vasodilation, which could be one of the possible mechanisms for the decreased SBP in CC-treated 2K/1C rats.

Histopathological examination of carotid arteries of vehicle-treated 2K/1C rats revealed increased smooth muscle cells proliferation and hypertrophy. We also observed a significant decrease in clipped kidneys’ weight in 2K/1C rats. eNOS-derived NO inhibits vascular growth by inactivating platelet-derived growth factor (28). Decreased eNOS expression and reduced NO content suggest that, it could be a causative factor for vascular smooth muscle cells proliferation, reduction in kidney weight, and hypertrophy in 2K/1C hypertensive rats. Cuminum cyminum treatment reversed these vascular changes, which could be substantiated to its restorative effect on eNOS and NO levels.
Increased TNF-α and IL-6 mRNA expressions in 2K/1C rats is a direct evidence of increased infiltration of inflammatory cells in clipped kidneys, and these events could be the consequence of alterations in NO production via angiotensin activation (29). TNF-α, a multifunctional circulating cytokine derived from endothelial and smooth muscle cells plays a primary role in the regulation of immune cells function (30). Preformed cytokines, specifically TNF-α, exist in various mast cell populations and this release can be induced by eosinophils and high-affinity IgE receptor (FceRI) ligations. Further, it induces the production of IL-6 in endothelial cells, fibroblasts, neutrophils, and mononuclear cells. Increased TNF-α and IL-6 in 2K/1C hypertensive rats could be responsible for tubular degeneration and interstitial mononuclear cell infiltrations. Cuminum cyminum suppressed TNF-α and IL-6 and increased eNOS expression in clipped kidneys, suggesting that it mediates anti-inflammatory effect via eNOS pathway in renal hypertensive rats.

Overexpression of TNF-α causes imbalance between pro-apoptotic (Bax) and antiapoptotic (Bcl-2) member of the Bcl-2 family (31). eNOS-derived NO regulates vascular structure by maintaining an antiproliferative and antiapoptotic environment in the vessel wall. Cuminum cyminum upholds the expression of Bax and Bcl-2 in renal tissues. This could be due to the increased production of nitric oxide and down-regulation of TNF-α and IL-6 expression in renal tissues of hypertensive rats.

TRX1 and TRXR1 are unique antioxidant systems that interact with various intracellular signaling molecules and are also key regulators of several transcriptional factors including NFκB-B (32). NFκ-B is involved in the transcription of TNF-α and IL-6 in primary substance P stimulated mast cells (33). It is well known that marked accumulation of superoxide in kidneys alters renal function and thus may contribute to the development of hypertension (34). Severe endothelial dysfunction and inhibition of microvascular nitric oxide synthase is accompanied by elevated reactive oxygen species. This inhibits and uncouples nitric oxide synthase and precedes the development of vascular hypertension (35,36). In our study, decreased plasma NO with arterial eNOS and renal TRX1 and TRXR1 mRNA down-regulation reflects the persistence of severe oxidative stress in 2K/1C rats. Further, this decreased TRX1 and TRXR1 would have activated NFκ-B, which in turn up-regulated TNF-α and IL-6 expressions (36). Increased TRX1 and TRXR1 levels demonstrate the ameliorative effect of CC against oxidative stress in hypertensive state.

Polyphenols, triterpenoids, and flavonoids are reported to slowdown the degenerative processes in several diseases such as cancer, heart disease, diabetes, and hypertension. Cuminum cyminum has been found to be rich in polyphenols; flavonoids; and triterpenoids such as cuminal, cumicin alcohol, γ-terpinene, p-cymene, and β-pinene (37,38). Dietary flavonoids and triterpenoids lower blood pressure and improve endothelial functions (39). Presence of considerable amount of polyphenols, flavonoids, and triterpenoids in CC might be the responsible chemical principles for the observed antihypertensive action.

CONCLUSION

To conclude, the anti-hypertensive activity exhibited by CC seeds is through the regulation of endothelial nitric oxide synthase and NO pathway with concomitant regulation of apoptosis, inflammation, and oxidative stress.

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Declaration of interest: The authors declare that they have no conflict of interest.

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