

Antihypertensive Effects of Diosmetin in Hypertension-Induced Cardiovascular Abnormalities in Rats

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Introduction: Hypertension is associated with cardiovascular dysfunction and remodeling. Diosmetin, a flavonoid isolated from citrus seeds, has various biological properties. This work investigated the effects of diosmetin on cardiovascular parameters and classical and non-classical renin-angiotensin systems (RAS) in two-kidney one-clip (2K-1C) rats.

Methods: 2K-1C hypertension was induced by placing a silver clip on the left renal artery. Three weeks after induction, rats were orally gavage with either vehicle, diosmetin (20 or 40 mg/kg), or telmisartan (5 mg/kg) for four weeks. Blood pressure (BP) was monitored weekly while vascular function and histomorphology studies were performed at the end of the study. Oxidative stress markers and RAS parameters, including serum angiotensin-converting enzyme (ACE) activity and plasma angiotensin II and angiotensin I-7 (Ang-(1-7)) concentrations, were also measured. The expression levels of angiotensin II type 1 receptor (AT₁R), transforming growth factor- β (TGF- β), and Mas receptor protein were assessed. A molecular docking analysis was performed to analyze the potential interactions between diosmetin and the human angiotensin I converting enzyme.

Results: In this in vivo study on the 2K-1C rats, diosmetin exhibited antihypertensive effects in the 2K-1C model via modulation of the RAS. Diosmetin at doses of 20 and 40 mg/kg decreased BP by 11.73% and 23.17%, respectively. Diosmetin also improved vascular function by reducing sympathetic nerve-mediated vasoconstriction and restoring endothelium-mediated vasodilation in the mesentery and aortic rings. The thickening of the left ventricle and aorta in hypertensive rats was alleviated by diosmetin treatment. RAS parameters and oxidative stress markers were improved in the diosmetin-treated group compared to the untreated group. Additionally, diosmetin treatment restored the overexpression of the AT₁R and TGF- β while reducing Mas receptor expression in cardiac and aortic tissue. The molecular docking analysis confirmed that diosmetin can bind to the active site of ACE.

Conclusion: Diosmetin restored hemodynamic alterations associated with the improvement of vascular function. It also ameliorated left ventricular-aortic hypertrophy in hypertensive rats. These effects could be attributed to its capacity to modulate classical and non-classical RAS.

Keywords: diosmetin, renovascular hypertensive rats, renin-angiotensin system, ACE inhibitor, vascular dysfunction, cardiovascular remodeling

Introduction

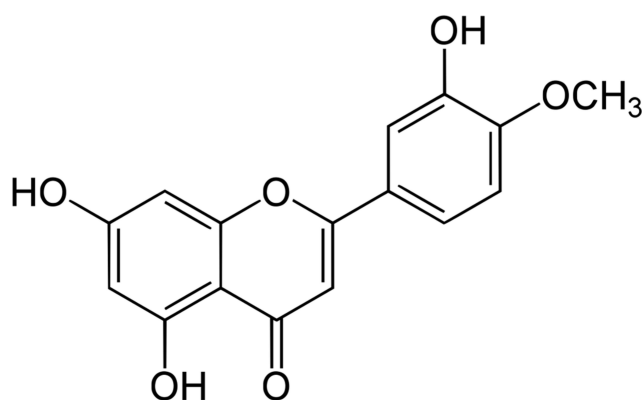
Hypertension is a chronic medical condition characterised by persistently elevated arterial blood pressure (BP), and is the primary cause of mortality and disability-adjusted life years worldwide.^{1,2} Secondary hypertension is a known cause and is mostly associated with kidney disease. Renovascular hypertension induced by the two-kidney, one-clip (2K-1C) method is a well-known animal model of angiotensin II (Ang II)-induced arterial hypertension.³ This procedure involves the partial



occlusion of a single side of the renal artery.⁴ Accumulating data suggest that activation of the renin-angiotensin system (RAS) exhibits a significant part in the induction and progression of renovascular hypertension.⁵ An increase in the activity of angiotensin-converting enzyme (ACE) and the level of Ang II have been observed in 2K-1C rats.^{6,7} Ang II acts on the angiotensin type 1 receptor (AT₁R) to induce vasoconstriction, promote cell growth and proliferation, and enhance sympathetic outflow.⁸ 2K-1C hypertension shows a high vascular tone due to sympathetic nerve-mediated vasoconstriction and vascular endothelial dysfunction.⁹ Left ventricular hypertrophy and enlargement of aorta have been seen in this animal model.^{10,11}

The molecular mechanism of Ang II-induced cardiac hypertrophy is associated with the activation of the AT₁R/transforming growth factor- β (TGF- β) pathway.¹² The TGF- β is a cytokine produced locally and a primary inducer of tissue fibrosis.¹³ It also regulates cell proliferation, differentiation, and migration.¹⁴ Ang II activates the AT₁R/TGF- β pathway, which is crucial for cardiac-myocyte hypertrophy, endothelial cells, and smooth muscle cells.¹⁵ A reduction of TGF- β protein expression can ameliorate vascular hypertrophy and remodelling in 2K-1C rats,^{16,17} while lowering TGF- β protein expression improves the cardiovascular morphology changes in 2K-1C rats.^{11,12} Moreover, Ang II also stimulated the generation of reactive oxygen species (ROS), which is a crucial cause of cardiovascular remodelling and dysfunction.¹⁶ ROS decrease the availability of nitric oxide (NO), resulting in cardiovascular disease.¹⁷ It is known that non-classical RAS can counteract classical RAS actions. Angiotensin-converting enzyme 2 (ACE2) converts Ang II to angiotensin 1–7 (Ang-(1-7)), which activates the Ang-(1-7) receptor, or Mas receptor (MasR), so counteracting the effects of AT₁R.¹⁸ Previous studies have reported that the Ang-(1-7)/MasR axis has vasodilatory, antihypertensive, antioxidant, anti-fibrotic, and anti-proliferative properties.^{14,15}

Diosmetin, *O*-methylated flavone, is a flavonoid glycoside isolated from citrus fruits (Figure 1).¹⁹ Several pharmacological properties of diosmetin have been reported, including antioxidant, anticancer, and anti-inflammatory.^{20,21} In alloxan-diabetic rats, diosmetin lowers blood glucose, triglycerides, and cholesterol.²¹ A prior study indicated that intravenous administration of diosmetin reduced mean arterial pressure in a dose-dependent fashion in both normotensive and hypertensive rats, with a more pronounced impact shown in hypertensive rats.²² In rats with metabolic syndrome, oral administration of diosmetin for four weeks was observed to lower blood pressure, which was connected to its antioxidant activity.²⁰ Diosmetin has remarkable antioxidant capabilities in aflatoxin B1-induced hepatotoxicity in mice by reducing lipid peroxidation and enhancing total antioxidant capacity.²³ Additionally, diosmetin reduces oxidative stress, inflammation, and enhances antioxidant enzymes via regulating nuclear factor erythroid 2-related factor 2 (Nrf-2)/haeme-oxygenase 1 (HO-1) and phosphorylated-c-Jun N-terminal kinase (p-JNK)/nuclear factor kappa B (NF- κ B) pathways.²⁴ In an L-NAME



The chemical structure of diosmetin

Figure 1 Chemical structure of diosmetin.

hypertensive rat model, diosmetin modulates Ca^{2+} channel antagonism to reduce hypertension.²² However, the mechanism by which diosmetin influences cardiovascular alterations during renovascular hypertension is unclear.

Therefore, this study aims to examine whether diosmetin reduces vascular dysfunction, left ventricular hypertrophy, and aortic hypertrophy in 2K-1C rats, focusing on mechanism of action of diosmetin on RAS and oxidative stress pathway. The hypothesis of this study is that diosmetin exhibits antihypertensive and antioxidant properties, which subsequently improve cardiovascular alteration in renovascular hypertension.

Material and Methods

Drugs and Animals

Diosmetin (98% HPLC) was obtained from Chengdu Biopurify Phytochemical Ltd. (Chengdu, China). Telmisartan (Micardis 80 mg) was obtained from Boehringer Ingelheim pharmaceuticals GmbH & Co., Inc. (Ingelheim am Rhein, Germany). Forty male Sprague-Dawley rats (5 weeks) from Nomura Siam International Co., Ltd. (Bangkok, Thailand) were used in this study. The animals were housed in a temperature-controlled room between $23 \pm 2^\circ\text{C}$ (heating, ventilation, and air-conditioning system with a 12 h dark-light cycle) at Northeast Laboratory Animal Center, Khon Kaen University, Khon Kaen, Thailand. All protocols were approved by the Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (IACUC-KKU-70/61), and complied with The Guide for the Care and Use of Laboratory Animals (NRC 2011; eighth edition) (<https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf>).

Induction of 2K-1C Hypertension and Experimental Designs

Forty male Sprague-Dawley rats (weighing 150–180 g) were randomly divided into normotensive sham-operative group and 2K-1C hypertensive group. After one week of acclimatisation, the rats were anaesthetised with xylazine (5 mg/kg, i.p.) and Zoletil (25 mg/kg, i.p.). Subsequently, the left renal artery was indicated and clipped with a silver clip (inner diameter, 0.2 mm) to create partial occlusion. The sham-operated group, done the same procedure except for clip insertion. Blood pressure was measured weekly, and sustained hypertension was noted 3 weeks after 2K-1C induction. Rats with systolic blood pressure (SBP) ≥ 160 mmHg were included in hypertension.²⁵ Thereafter, the rats were randomly divided into five groups ($n=8/\text{each}$): the sham-operated and 2K-1C group, which received propylene glycol (PG, 1.5 mL/kg) as a vehicle, and the 2K-1C-treated group, which received diosmetin (20 and 40 mg/kg) or telmisartan (5 mg/kg). Daily administration of diosmetin, telmisartan, and PG was conducted for four weeks via a gavage feeding tube. The dosages of diosmetin and telmisartan employed in this investigation were informed by prior research.^{26–28} Experimental designs are shown in Figure 2.

Blood Pressure Monitoring

SBP was measured in conscious rats weekly using the tail-cuff method (CODA® mouse rat tail-cuff system, Kent Scientific Co., CT, USA). All rats were trained to acclimatise to the system before blood pressure measurements. The average of 15 measured values from each rat was used.

Assessment of Hemodynamic Parameters

A direct method for blood pressure assessment was made under anesthesia (with thiopental sodium, 60 mg/kg i.p.) via left femoral artery. Thirty minutes of data recording were analyzed using Acknowledge Data Acquisition software (Biopac Systems Inc., Santa Barbara, CA, USA) to obtain hemodynamic parameters. The data of SBP, diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP), and heart rate (HR) were collected. Subsequently, blood samples were obtained, and the rats were euthanized via exsanguination. Organ tissues were acquired.

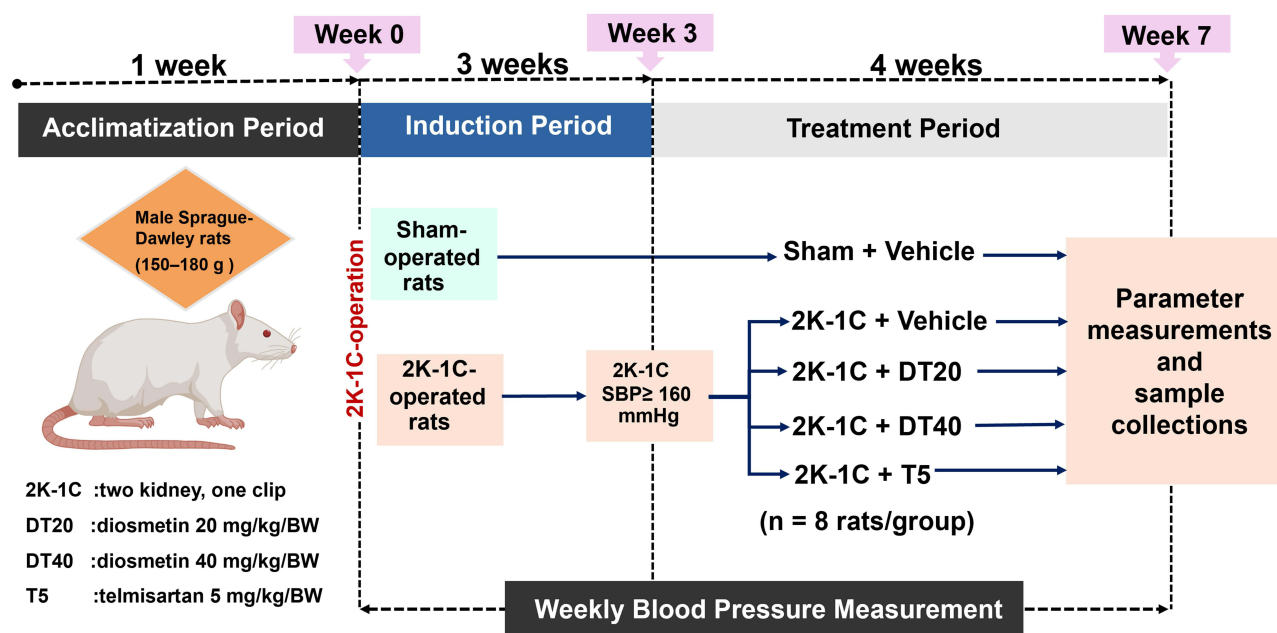


Figure 2 Experimental designs of the study. 2K-1C; two-kidney, one clip, DT20; diosmetin (20 mg/kg), DT40; diosmetin (40 mg/kg), T5; telmisartan (5 mg/kg).

Assessment of Vascular Function

Contractile responses to sympathetic nerve stimulation and exogenous norepinephrine and relaxation responses to vasoactive agents were evaluated in the mesenteric beds of all groups of rats. The mesenteric vascular beds were rapidly isolated, set up with a pressure transducer, and perfused with physiological Krebs solution for 30 min before starting the experiment. The contractile response to an electrical field stimulation (EFS, 5–40 Hz, 90 V, and 1 ms for 30s) and exogenous norepinephrine (NE, 0.15–15 nmol) were performed, respectively. After that, the vascular tone of the mesenteric bed was raised by an α -1 agonist, methoxamine hydrochloride (5–7 μ M), before a bolus injection of acetylcholine (ACh, 0.1 to 10 nmol) and sodium nitroprusside (SNP, 0.1 to 10 nmol) to assess the vasorelaxation response of the artery. Moreover, the vasorelaxation response to ACh (0.01–3 μ M) or SNP (0.01–3 μ M) was also evaluated in aortic rings. The aorta was collected, and connective tissue was removed before setting up in the organ bath chambers. After a resting period, the vascular tone was raised with an α -1 agonist, phenylephrine, before cumulatively adding ACh or SNP into the bath.

Histological Examination

Left ventricular tissue and thoracic aorta were fixed in 10% formalin solution for 24 h, then these tissues were dehydrated and cleared in serial alcohol dilutions and xylene. Subsequently, these tissues were normally treated in paraffin, followed by the preparation of serial slices with a thickness of 5 μ m, which were stained using hematoxylin and eosin (Bio-Optica, Milano SpA., Milano, Italy). Vascular images were obtained under the normal light mode using an Eclipse Ci-POL polarized light microscope with 20X objective lenses (Nikon, Tokyo, Japan). The left ventricular images were obtained using a stereomicroscope with 2X objective lenses. ImageJ morphometric software (National Institutes of Health, Bethesda, MD, USA) was used for morphometric evaluations, including wall thickness, cross-sectional area, and luminal area.

Biochemical Assays

To minimize bias, all biochemical assays were performed by an investigator blinded to the experimental group assignments. Samples were coded and the key was kept sealed until after data analysis. Serum ACE activity was assayed in all groups using a fluorescence assay.⁶ The mixture of the serum and assay buffer was incubated at 37°C for 30 min before

adding 150 μ L of 0.1 M NaOH to stop the activity. The product of the reaction was labelled with 10 mg/mL o-phthaldialdehyde, and ACE activity (mU/mL) was measured using a microplate reader at a wavelength of 390 nm. In addition, the levels of Ang-(1-7) and Ang II was identified in the plasma according to the established protocol guidelines of an Ang-(1-7) ELISA Kit (CSB-E14241r; CUSABIO TECHNOLOGY, Texas, USA) and an Ang II Enzyme Immunoassay Kit (RAB0010; Sigma-Aldrich, St. Louis, MO, USA), respectively.

Assessment of Oxidative Markers

The level of superoxide production ($O_2^{\cdot-}$) was determined in the aorta, heart, and kidney tissues using the lucigenin-enhanced chemiluminescence method,²⁹ with some modifications.³⁰ Plasma malondialdehyde (MDA) concentrations were detected using thiobarbituric acid-reactive substances (TBARS).³¹ Catalase (CAT) activity was assessed with a spectrophotometric technique, as previously documented.³²

Protein Expression Detection

The expression levels of AT1R, MasR, and TGF- β proteins in cardiac and aortic tissues were evaluated using the Western blot technique. Tissue samples were subjected to electrophoresis using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% bovine serum albumin at room temperature (25°C) for 2 h before overnight incubation with primary antibodies against AT1R (sc-515884, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), MasR (sc-390453, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and TGF- β (sc-52893, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C. After incubation, the membranes were washed twice before incubation with a secondary antibody for 2 h at room temperature. Band signals were developed using chemiluminescence (ECL) kits in a digital imaging system for sensitive, quantitative imaging of gels and blots (ImageQuant™ LAS 4000, GE Healthcare Life Science, Piscataway, NJ, USA). The intensity of protein bands was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA) and normalized to its β -actin.

Molecular Docking

Molecular docking was evaluated as previously reported³³ with modifications. Briefly, the 3D structure of diosmetin (CID5281612) was obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>) and subsequently energy-minimized in PyRx (PyRx-Python Prescription 0.8) employing the Merck Molecular Force Field (MMFF94). The 3D crystal structure of human angiotensin I converting enzyme (PDB ID: 1UZE) was retrieved from the Protein Data Bank and preprocessed using AutoDockTools (version 1.5.7), which involved the removal of water molecules and co-crystallized ligands, followed by the addition of polar hydrogens and assignment of charges. The prepared ligand was then docked into the active site of the processed enzyme using PyRx, and the resulting binding poses were visualized with PyMOL (version 2.4.1; PyMOL Molecular Graphics System, Schrödinger) while the interactions were further determined using Discovery Studio Visualizer (version 21.1.0.20298; BIOVIA, San Diego, CA, USA).

Statistical Analysis

The results are presented as means $\pm$ SEM. A one-way analysis of variance (ANOVA) accompanied by Tukey's post-hoc test was employed to compare the groups. Statistical significance was established as a probability (p) value below 0.05. All statistical analyses were conducted using GraphPad Prism 10.0 (GraphPad Software Inc., Boston, MA, USA).

Results

Impact of Diosmetin on Blood Pressure

The blood pressure among all rat groups at baseline (week 0) was comparable ([Supplementary Data](#)). The effects of left renal artery clipping and diosmetin supplementation on SBP were monitored weekly throughout the seven weeks of experimental periods ([Figure 3](#)). A dose-dependent effect of diosmetin on SBP reduction was found in hypertensive rats

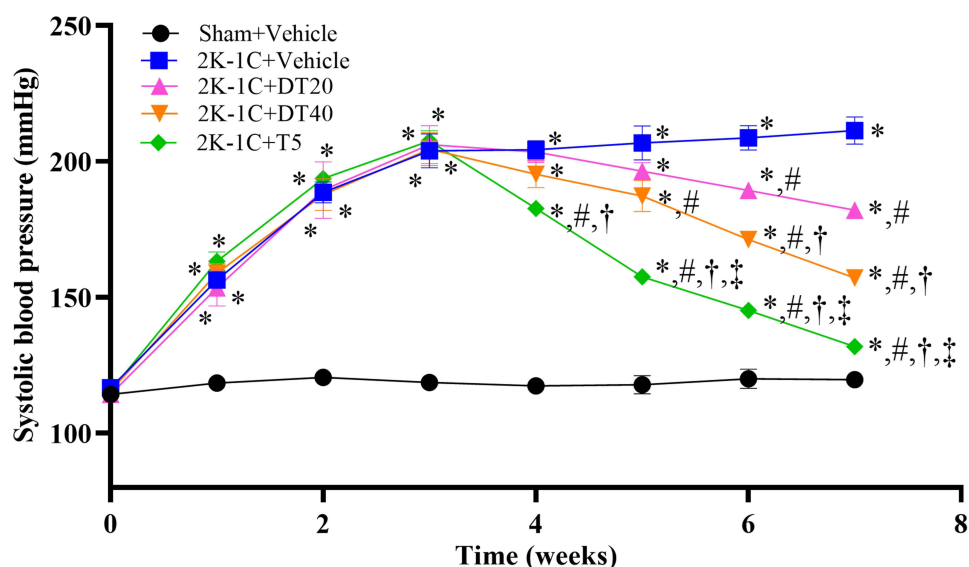


Figure 3 Systolic blood pressure (SBP) in all groups. The data are expressed as mean $\pm$ SEMs. 2K-1C; two-kidney, one clip, DT20; diosmetin (20 mg/kg), DT40; diosmetin (40 mg/kg), T5; telmisartan (5 mg/kg). * $p < 0.05$ versus Sham + Vehicle, # $p < 0.05$ versus 2K-1C + Vehicle, † $p < 0.05$ versus 2K-1C + DT20, ‡ $p < 0.05$ versus 2K-1C + DT40.

compared to vehicle-treated rats ($p < 0.0001$). In addition, treatment with telmisartan (5 mg/kg) resulted in the greatest reduction in SBP compared to that in vehicle-treated rats ($p < 0.0001$).

Hemodynamic parameters were obtained under anesthesia at the end of the experimental day (Table 1). A significant increase in hemodynamic parameters was observed in 2K-1C untreated rats compared to sham rats ($p < 0.0001$). Supplementation with diosmetin significantly reduced this alteration compared to that in untreated rats and it showed a dose-dependent effect on SBP, DBP, and MAP, but not on PP and HR. In addition, treatment with telmisartan showed antihypertensive effects in 2 K-1C hypertensive rats, and this effect was significantly greater than that of diosmetin, as shown in Table 1.

Impact of Diosmetin on Body Weight and Organ Weights

At the end of the experiment, a significant reduction in body weight (BW) was observed in a 2K-1C group compared to that in a sham group ($p = 0.0002$; Table 2). In 2K-1C hypertensive rats, diosmetin (40 mg/kg) or telmisartan treatment markedly reduced BW ($p < 0.05$). Additionally, the organ weight/BW ratios were significantly higher in a 2K-1C group than in a sham group ($p < 0.0001$). After administering diosmetin (40 mg/kg) or telmisartan, the increase in these ratios was attenuated in 2K-1C rats, as shown in Table 2.

Table 1 Hemodynamic Parameters in All Groups of the Experiments

Parameters	Sham	2K-1C			
	Vehicle	Vehicle	DT20	DT40	T5
SBP (mmHg)	123.61 $\pm$ 1.71	201.84 $\pm$ 4.86*	179.06 $\pm$ 1.86* [#]	157.80 $\pm$ 2.12* ^{#,†}	131.21 $\pm$ 4.61 ^{#,†,‡}
DBP (mmHg)	73.22 $\pm$ 2.30	145.69 $\pm$ 3.19 *	120.29 $\pm$ 8.28 * [#]	95.81 $\pm$ 4.53 * ^{#,†}	78.37 $\pm$ 3.99 ^{#,†,‡}
MAP (mmHg)	90.74 $\pm$ 1.72	159.54 $\pm$ 3.77*	129.90 $\pm$ 6.52 * [#]	111.16 $\pm$ 3.54 * ^{#,†}	96.17 $\pm$ 3.00 ^{#,†,‡}
PP (mmHg)	43.29 $\pm$ 1.25	72.99 $\pm$ 1.66 *	66.50 $\pm$ 4.03 *	56.94 $\pm$ 3.08 * [#]	47.76 $\pm$ 1.80 ^{#,†}
HR (beats/min)	345.63 $\pm$ 2.36	427.27 $\pm$ 9.47 *	406.35 $\pm$ 7.50 *	375.40 $\pm$ 10.18* [#]	364.76 $\pm$ 10.29 ^{#,†}

Notes: The results are shown as mean $\pm$ standard error of the means (SEM). * $p < 0.05$ versus Sham + Vehicle, # $p < 0.05$ versus 2K-1C + Vehicle, † $p < 0.05$ versus 2K-1C + DT20, ‡ $p < 0.05$ versus 2K-1C + DT40.

Abbreviations: SBP, Systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; HR, heart rate; 2K-1C, two-kidney, one clip; DT20, diosmetin (20 mg/kg); DT40, diosmetin (40 mg/kg); T5, telmisartan (5 mg/kg).

Table 2 Effects of Diosmetin or Telmisartan on Body Weight

Parameters	Sham	2K-1C			
	Vehicle	Vehicle	DT20	DT40	T5
BW (g)	551.63 ± 8.04	448.14 ± 18.54*	469.75 ± 17.72*	521.14 ± 17.11 [#]	516.14 ± 9.91 [#]
HW/BW (mg/g)	2.76 ± 0.07	4.06 ± 0.21*	3.90 ± 0.18*	3.36 ± 0.15 [#]	3.25 ± 0.04 ^{#,†}
VW/BW (mg/g)	2.24 ± 0.03	3.65 ± 0.16*	3.41 ± 0.15*	2.95 ± 0.14 [#]	2.51 ± 0.11 ^{#,†}
LVW/BW (mg/g)	1.74 ± 0.02	2.92 ± 0.14*	2.83 ± 0.13*	2.47 ± 0.13 [#]	2.27 ± 0.10 ^{#,†}
RKW/BW (mg/g)	3.30 ± 0.05	5.92 ± 0.23*	5.27 ± 0.18*	4.78 ± 0.20 [#]	4.68 ± 0.23 [#]
LKW/BW (mg/g)	3.39 ± 0.03	2.79 ± 0.04*	2.80 ± 0.13*	2.84 ± 0.013*	2.78 ± 0.12*

Notes: The results are shown as mean ± standard error of the means (SEM). * $p < 0.05$ versus Sham + Vehicle, [#] $p < 0.05$ versus 2K-1C + Vehicle, [†] $p < 0.05$ versus 2K-1C + DT20.

Abbreviations: HW/BW, heart weight/body weight ratio; VW/BW, ventricular weight/body weight ratio; LVW/BW, left ventricular weight/body weight ratio; RKW/BW, right kidney weight/body weight ratio; LKW/BW, left kidney weight/body weight; 2K-1C, two-kidney, one clip; DT20, diosmetin (20 mg/kg); DT40, diosmetin (40 mg/kg); T5, telmisartan (5 mg/kg).

Impact of Diosmetin on Sympathetic Nerve-Mediated Vasoconstriction and Exogenous Norepinephrine Injection

For all preparations, a frequency-dependent contractile response to EFS was observed in the mesenteric vascular bed (MVB) (Figure 4A). In the preparation isolated from 2K-1C rats, the vasoconstriction response to EFS was greater than that in sham rats ($p < 0.0001$). Interestingly, the response to EFS was much lower in rats treated with diosmetin and telmisartan than in 2K-1C hypertensive rats ($p < 0.0001$). In addition, the contractile response to exogenous NE (0.15–15 nM) was comparable in all preparations (Figure 4B).

Impact of Diosmetin on Vascular Endothelial Function

A significant decrease in the vasorelaxation response to ACh was seen in the mesenteric vascular bed isolated from a hypertensive group compared to that in a sham group ($p < 0.0001$). Diosmetin and telmisartan treatment improved the response to ACh compared to the vehicle-treated group ($p < 0.0001$) (Figure 5A). Consequently, the response to the SNP remained comparable across the groups (Figure 5B). Similar results were found in the aortic ring setup; there was a significant decrease in vasorelaxation in the aortic rings from the 2K-1C hypertensive group compared to that in the

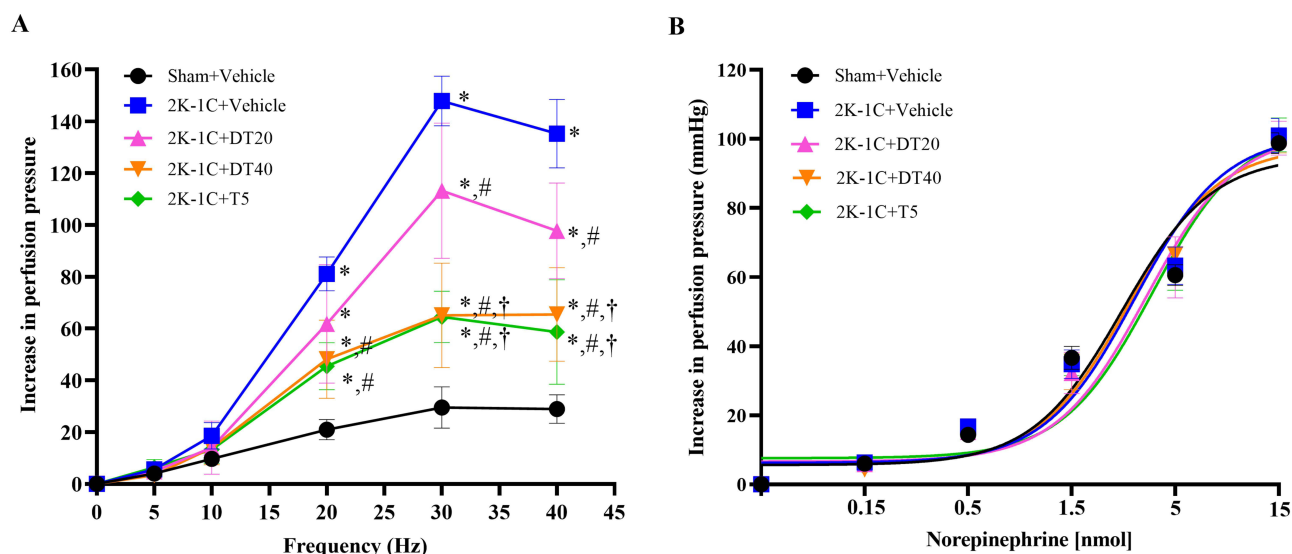


Figure 4 The vasoconstriction responses to an electrical field stimulation (A) and norepinephrine (B) in the mesenteric bed of all experimental groups. The results are depicted as mean ± SEMs. 2K-1C, two-kidney, one clip; DT20, diosmetin (20 mg/kg); DT40, diosmetin (40 mg/kg); T5, telmisartan (5mg/kg). * $p < 0.05$ versus Sham + Vehicle, [#] $p < 0.05$ versus 2K-1C + Vehicle, [†] $p < 0.05$ versus 2K-1C + DT20.

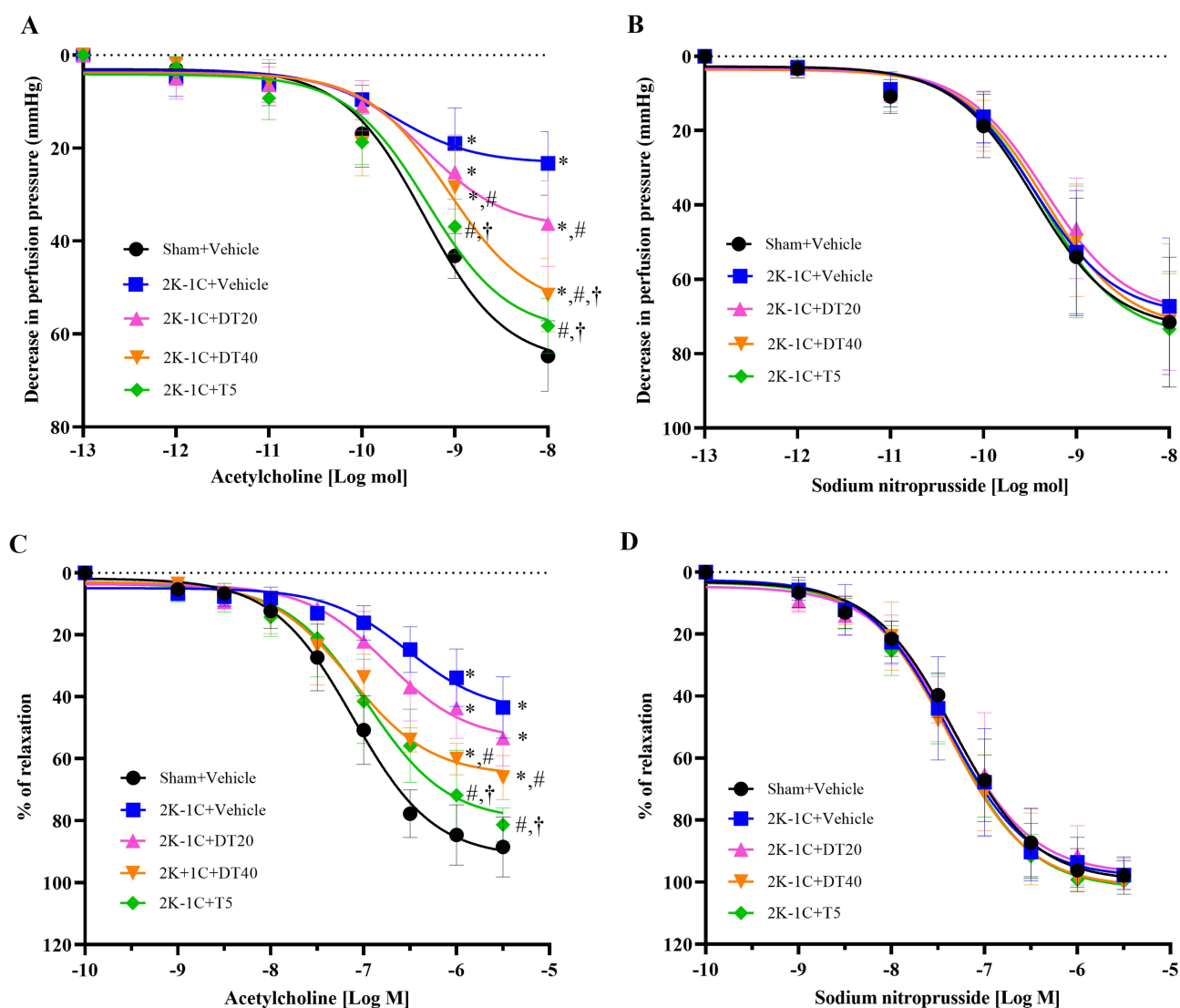


Figure 5 The responses to acetylcholine (A) and sodium nitroprusside (B) in the mesenteric bed and acetylcholine (C) and sodium nitroprusside (D) in aortic rings. The data are expressed as mean $\pm$ SEMs. 2K-1C, two-kidney, one clip; DT20, diosmetin (20 mg/kg); DT40, diosmetin (40 mg/kg); T5, telmisartan (5mg/kg). * $p < 0.05$ versus Sham + Vehicle, # $p < 0.05$ versus 2K-1C + Vehicle, † $p < 0.05$ versus 2K-1C + DT20.

sham group ($p < 0.0001$). Supplementation with diosmetin at a dose of 40 mg/kg or telmisartan improved the vasorelaxation response to ACh compared to the untreated group ($p = 0.0103$, and $p = 0.0001$ respectively) (Figure 5C). Consequently, the response to the SNP remained comparable across the groups (Figure 5D).

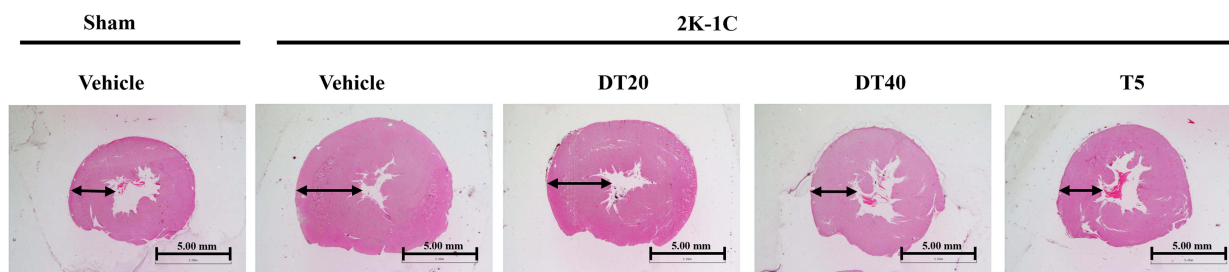
Impact of Diosmetin on Cardiac Morphology

In 2K-1C hypertensive rats, LV wall thickness and cross-sectional area were found to increase compared to that in sham rats ($p < 0.0001$). In addition, a reduction in the LV luminal area was seen in the vehicle-treated hypertension compared to a sham group ($p < 0.0001$), diosmetin (40 mg/kg) or telmisartan treatment reduced cardiac changes in hypertensive rats, as shown in Figure 6A–D.

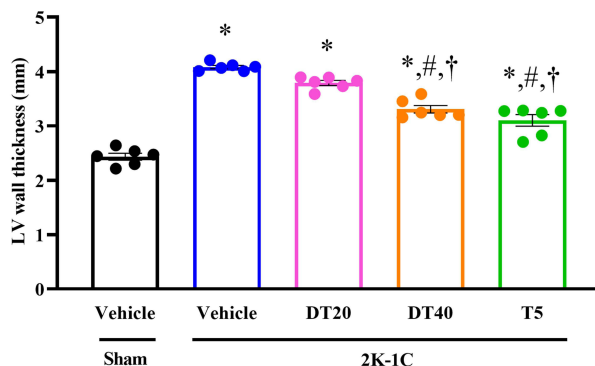
Impact of Diosmetin on Vascular Morphology

A significant increase in vascular wall thickness, cross-sectional area, and wall/lumen ratios was seen in the hypertensive group compared to the control rats (Figure 7A–C and E) ($p < 0.0001$). Treatment with diosmetin (40 mg/kg) or telmisartan

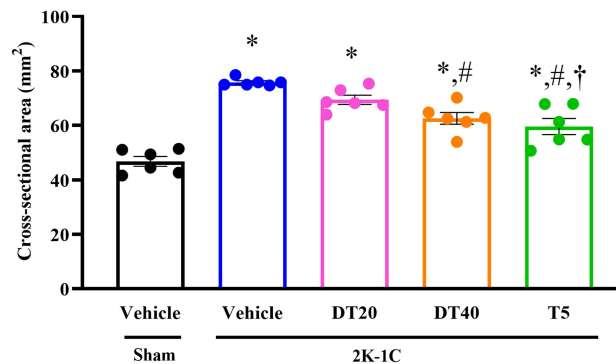
A



B



C



D

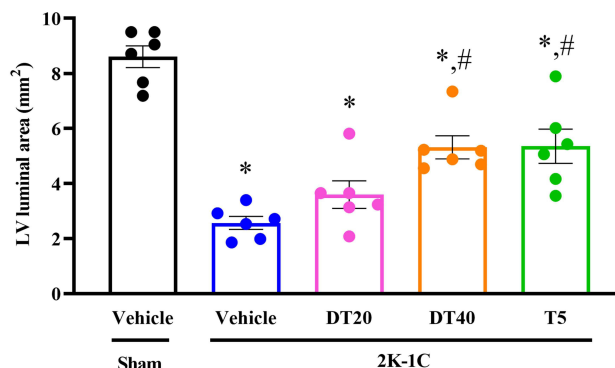


Figure 6 Representative image of cardiac tissue stained with H&E (Double-headed arrow indicates cardiac wall thickness in all groups of experiments) (A). The semiquantitative assessment of cardiac remodeling is shown by left ventricular wall thickness (B), cross-sectional areas (C), and LV luminal areas (D). The data are expressed as mean $\pm$ SEMs. 2K-1C, two-kidney, one clip; DT20, diosmetin (20 mg/kg); DT40, diosmetin (40 mg/kg); T5, telmisartan (5mg/kg). * $p < 0.05$ versus Sham + Vehicle, # $p < 0.05$ versus 2K-1C + Vehicle, † $p < 0.05$ versus 2K-1C + DT20.

markedly reduced this alteration in aortic tissue collected from 2K-1C rats. Nevertheless, the luminal diameters exhibited no differences between the groups (Figure 7D).

Impact of Diosmetin on RAS Parameters (ACE Activity, Ang II, and Ang-(1-7) Levels)

High concentrations of serum ACE activity and plasma Ang II were found in hypertensive rats compared to those in a sham group ($p < 0.0001$). Diosmetin supplementation at a dose of 40 mg/kg or telmisartan attenuated RAS overactivity, as indicated by a significant decrease in serum ACE activity and plasma Ang II levels induced by 2K-1C as shown in Figure 8A and B. In addition, untreated 2K-1C rats had significantly decreased plasma Ang-(1-7) levels compared to

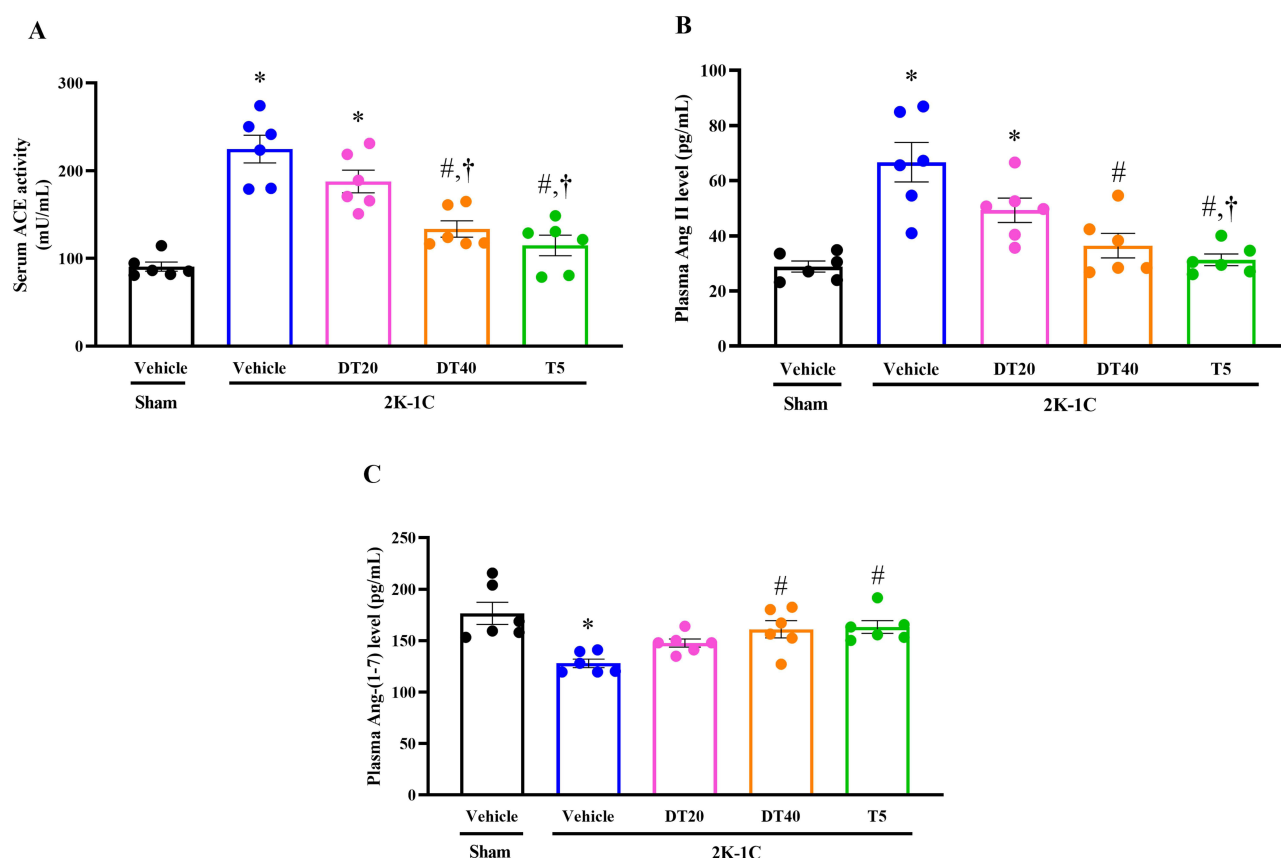


Figure 8 Serum angiotensin-converting enzyme (ACE) activity (A), plasma angiotensin II (Ang II) levels (B), and plasma angiotensin 1–7 (Ang-(1-7)) levels (C). The data are expressed as mean $\pm$ SEMs. 2K-1C, two-kidney, one clip; DT20, diosmetin (20 mg/kg); DT40, diosmetin (40 mg/kg); T5, telmisartan (5mg/kg). * $p < 0.05$ versus Sham + Vehicle, # $p < 0.05$ versus 2K-1C + Vehicle, † $p < 0.05$ versus 2K-1C + DT20.

Impact of Diosmetin on the Expression of AT₁R, TGF- β , and MasR Protein in Cardiac Tissue

The expression of AT₁R and TGF- β proteins in the cardiac tissue collected from hypertensive rats was increased compared to that in the control ($p < 0.002$). Diosmetin (40 mg/kg) or telmisartan restores the overexpression of AT₁R and TGF- β proteins compared to vehicle-treated hypertensive rats ($p < 0.01$). MasR protein expression was significantly downregulated in cardiac tissue of hypertensive rats compared to that in the control rats ($p = 0.0016$). Nevertheless, diosmetin or telmisartan improved the expression of MasR protein compared to that in the untreated rats as shown in Figure 10.

Impact of Diosmetin on the Expression of AT₁R, TGF- β , and MasR Protein in Aortic Tissue

Overexpression of AT₁R and TGF- β proteins has been found in the aortic tissue collected from hypertensive rats compared to that in the control rats ($p < 0.003$). Diosmetin (40 mg/kg) or telmisartan significantly restored these alterations compared to untreated rats ($p < 0.005$). MasR protein expression was significantly downregulated in untreated rats, whereas supplementation with diosmetin (40 mg/kg) or telmisartan significantly restored MasR expression as shown in Figure 11.

Impact of Diosmetin on Molecular Interactions Between Diosmetin and ACE

Molecular docking analysis was conducted to evaluate the potential interactions between diosmetin and human angiotensin I converting enzyme (PDB ID: 1UZE). The results revealed that diosmetin binds to ACE with an affinity of -8.6

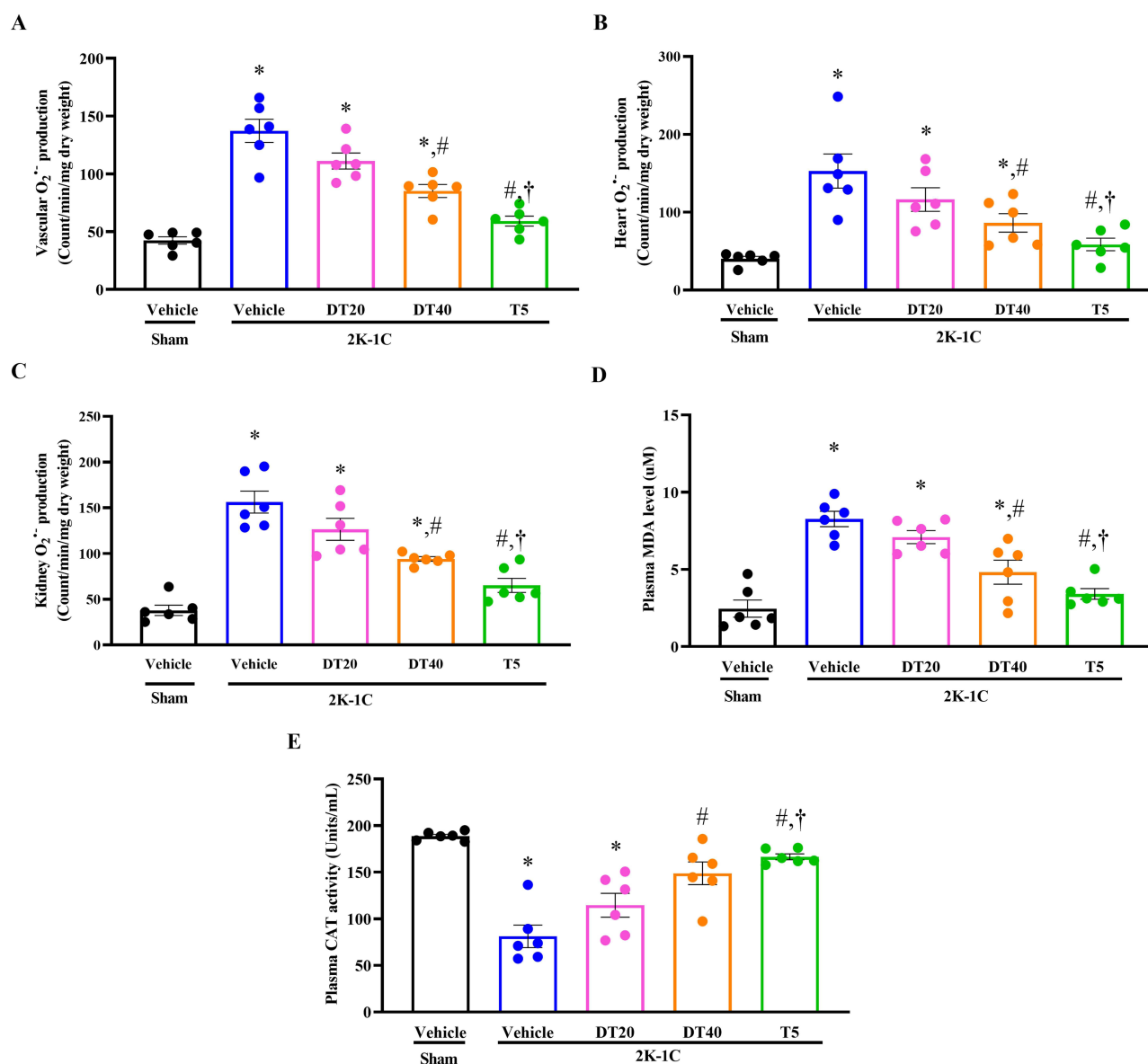


Figure 9 Effects of diosmetin or telmisartan on O_2^- production in the vascular (A), heart (B), kidney (C), and plasma malondialdehyde (MDA) levels (D). Levels of plasma catalase (CAT) activity (E) in all groups of rats. The data are expressed as mean $\pm$ SEMs. 2K-1C, two-kidney, one clip; DT20, diosmetin (20 mg/kg); DT40, diosmetin (40 mg/kg); T5, telmisartan (5mg/kg). * $p < 0.05$ versus Sham + Vehicle, # $p < 0.05$ versus 2K-1C + Vehicle, † $p < 0.05$ versus 2K-1C + DT20.

kcal/mol at the catalytic domain, which includes the zinc-binding motif. Notably, the docking pose of diosmetin superimposes with the experimental binding conformation of enalaprilat, a well-established ACE inhibitor (Figure 12A), suggesting a similar mechanism of action. Further analysis demonstrated that the interactions between diosmetin and ACE are primarily mediated by hydrophobic forces, complemented by the formation of two hydrogen bonds (Figure 12B and C). In contrast, enalaprilat exhibits a stronger binding profile by forming four hydrogen bonds and two ionic bonds (Figure 12D), which may explain its superior efficacy. Collectively, these findings indicate that diosmetin is capable of binding to the catalytic site of ACE, although its overall binding effectiveness may be lower than that of enalaprilat.

Discussion

The results indicated that diosmetin significantly reduced blood pressure in 2K-1C rats. It also improved the vascular response to acetylcholine, an endothelium-dependent vasoactive agent, and reduced the vasoconstriction responses to

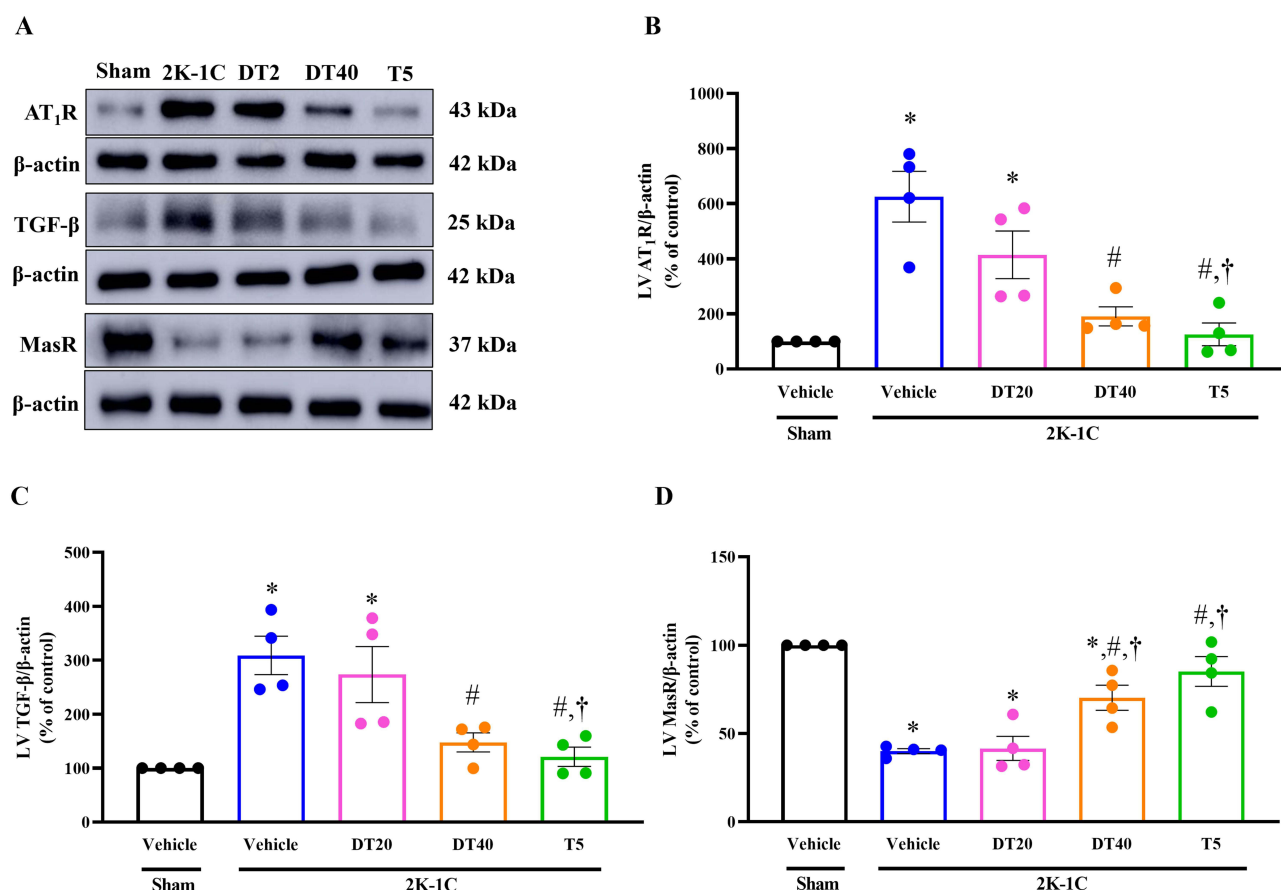


Figure 10 Representative Western blot images of membranes detected with chemiluminescence (A). Effects of diosmetin or telmisartan on AT₁R (B), TGF-β (C), and MasR (D) protein expression in cardiac tissue collected from 2K-1C hypertensive rats. The data are expressed as mean ± SEMs. 2K-1C, two-kidney, one clip; DT20, diosmetin (20 mg/kg); DT40, diosmetin (40 mg/kg); T5, telmisartan (5 mg/kg). * $p < 0.05$ versus Sham + Vehicle, # $p < 0.05$ versus 2K-1C + Vehicle, † $p < 0.05$ versus 2K-1C + DT20.

sympathetic nerve stimulation in 2K-1C rats. 2K-1C rats treated with diosmetin showed reduced cardiac and vascular hypertrophy. High levels of serum ACE activity, plasma Ang II, and oxidative stress were alleviated by diosmetin treatment. It suppressed AT₁R/TGF-β overexpression in both cardiac and aortic samples but enhanced plasma Ang-(1-7) levels and MasR expression in the tissue. We used telmisartan, a positive control agent, and when comparing the doses utilized, telmisartan had a stronger effect than diosmetin.

It is well established that in a 2K-1C model, elevated blood pressure is primarily caused by RAS activation.^{34,35} In the present study, diosmetin ameliorated high blood pressure and haemodynamic changes in renovascular hypertensive rats. The results indicated that 40 mg/kg diosmetin was the most effective dose in the present study. The antihypertensive properties of diosmetin may be associated with decreased ACE activity and Ang II levels. These findings are consistent with several publications that diosmetin has ACE inhibitory and Ang II level-lowering effects.^{20,36} Additionally, diosmetin increased Ang-(1-7) levels and upregulated MasR protein expression in hypertensive rats, which is a non-classical RAS axis. Non-classic RAS functions as an endogenous counterregulatory arm of the Ang II/ACE axis, including reducing blood pressure.³⁷ Our findings were corroborated by prior research indicating that flavonoids, including baicalin and chrysin, can activate the Ang (1-7)/MasR axis.^{38,39}

Interestingly, diosmetin also demonstrates antioxidant properties by lowering MDA, an indicator of lipid peroxidation, and increasing the activity of the endogenous antioxidant enzyme CAT. In the 2K-1C rat model, considerable evidence suggests that oxidative stress is essential for sustaining elevated blood pressure.⁴⁰ Substantial evidence showed antioxidant activities under different conditions.^{41,42} A recent study by Wójciak et al in 2022, demonstrated that diosmin/diosmetin alleviated H₂O₂-induced oxidative stress, restored the activity of SOD and CAT, and reduced MDA levels,

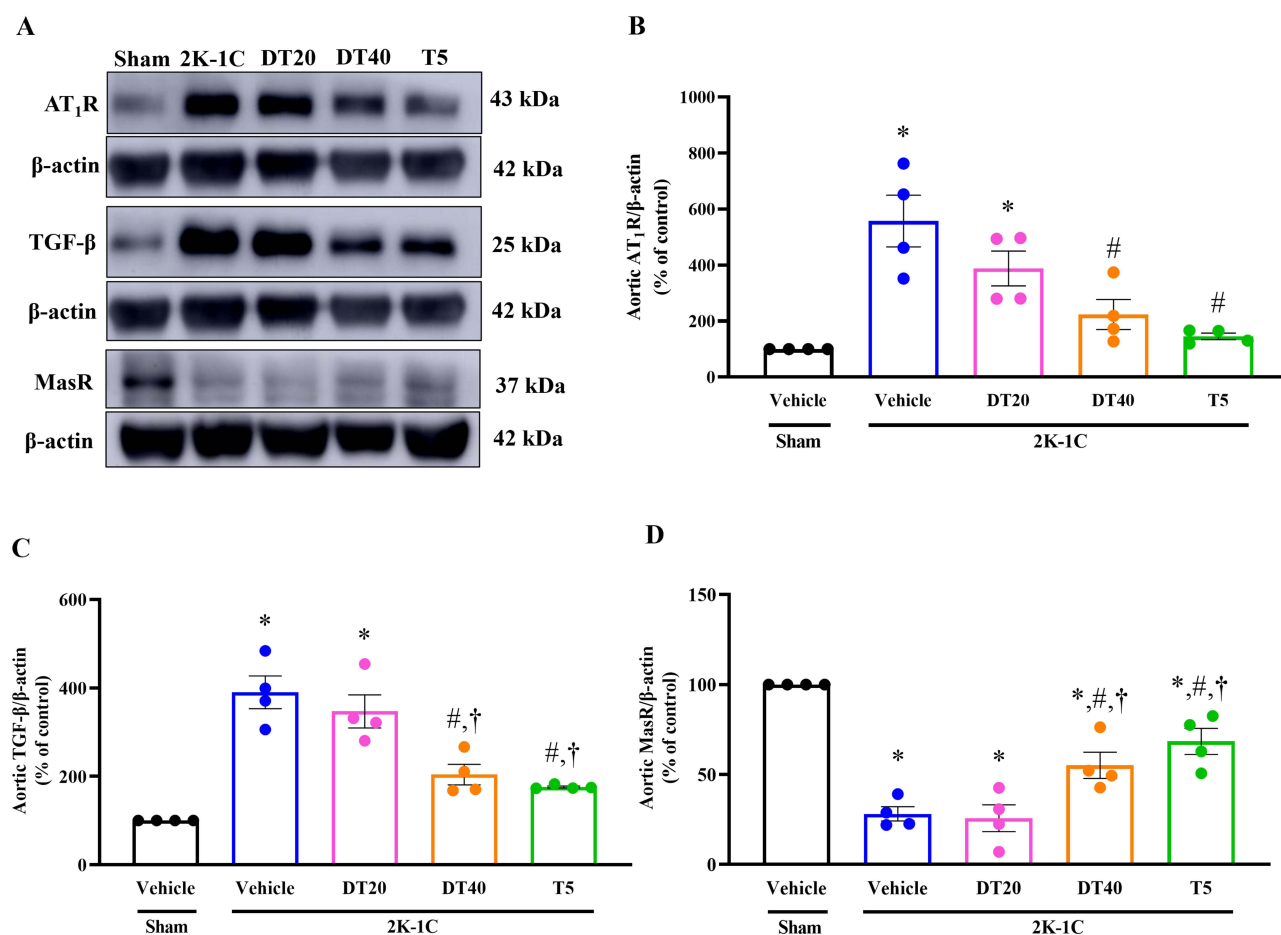


Figure 11 Representative Western blot images of membranes detected with chemiluminescence (A). Effects of diosmetin or telmisartan on AT₁R (B), TGF-β (C), and MasR (D) protein expression in aorta tissue collected from all groups. The data are expressed as mean ± SEMs. 2K-1C, two-kidney, one clip; DT20, diosmetin (20 mg/kg); DT40, diosmetin (40 mg/kg); T5, telmisartan (5mg/kg). * $p < 0.05$ versus Sham + Vehicle, # $p < 0.05$ versus 2K-1C + Vehicle, † $p < 0.05$ versus 2K-1C + DT20.

suggesting that diosmin and diosmetin could prevent oxidative stress in endothelial cells and thus protect against the development and progression of oxidative stress-related disorders.⁴¹

Deterioration of the vascular function was observed in vessels isolated from 2K-1C hypertensive rats. This vascular function impairment might have been caused by excessive sympathetic nervous system activity, supported by the elevated vascular response to EFS, but not by exogenous norepinephrine injection. These results align with those of numerous studies that have demonstrated elevated sympathetic activity in 2K-1C hypertensive rats.^{43,44} Furthermore, studies have linked sympathetic neuron overactivity with endothelial dysfunction.^{45,46} Interestingly, the vascular response to acetylcholine was reduced, indicating abnormal endothelial cell function and vascular dysfunction. It has been reported that the activation of RAS, $O_2^{\bullet-}$, and depletion of NO production in a 2K-1C rat model contribute to vascular dysfunction.⁴⁵ Therefore, diosmetin may mitigate vascular dysfunction in 2K-1C hypertensive rats via mechanisms related to the suppression of RAS, $O_2^{\bullet-}$, and sympathetic production. Diosmetin also increased the expression of the Ang-(1-7)/MasR signalling pathway in the vascular tissue of 2K-1C hypertensive rats. Ang-(1-7)/MasR signalling is related to NO production and antioxidant activity and counteracts the ACE/Ang II/AT₁R signalling pathway.^{47,48} Thus, molecular processes involving activation of the Ang-(1-7)/MasR signalling pathway and suppression of the ACE/Ang II/AT₁R signalling pathway in 2K-1C rats may mediate the positive effects of diosmetin on vascular function found in this study. Interestingly, Ahmad et al provide strong evidence that ties the precise mechanisms of diosmetin's antihypertensive action via the improvement of vascular function. In vivo study, diosmetin's antihypertensive effect is linked to the muscarinic receptor rather than NO. In vitro study, diosmetin-induced vasorelaxation is comparable to verapamil in aortic

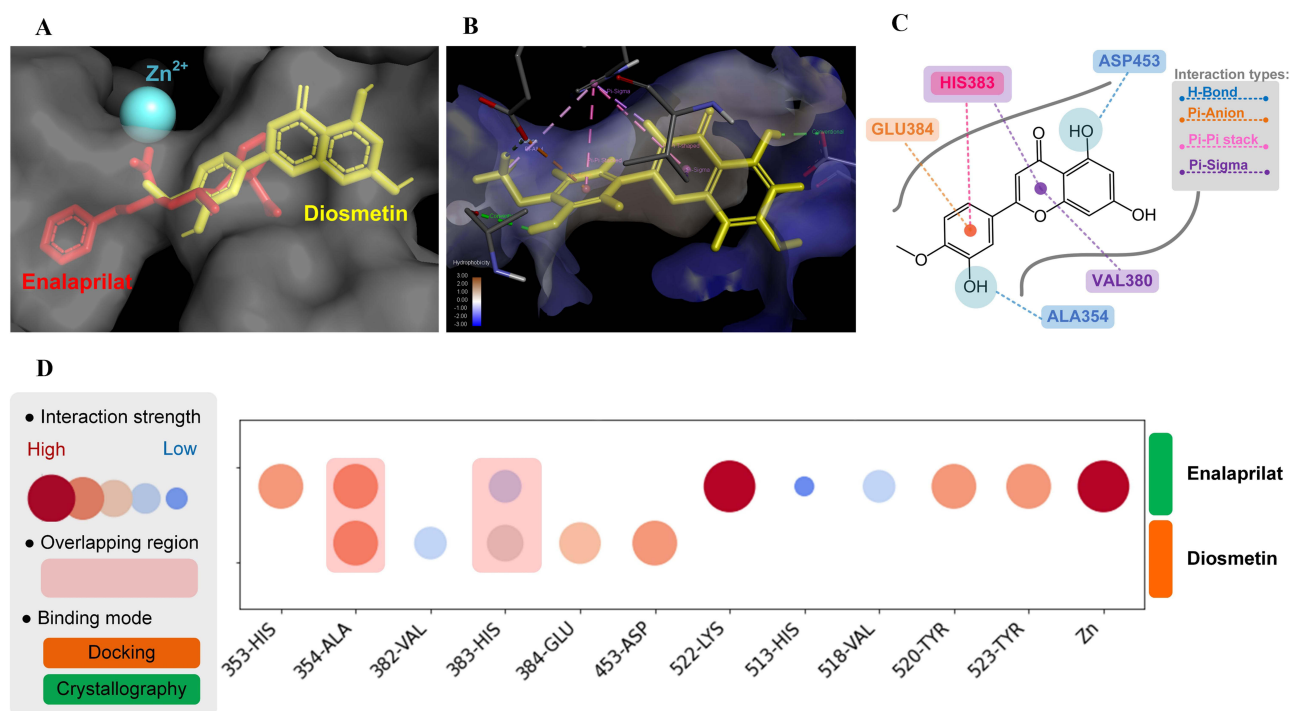


Figure 12 Molecular interactions between diosmetin and human ACE (PDB ID: 1UZE). **(A)** Superimposition of the docking pose of diosmetin and the crystallographic pose of enalaprilat at the catalytic domain. **(B)** Three-dimensional visualization of diosmetin binding at the active site. **(C)** Two-dimensional diagram summarizing key molecular interactions. **(D)** Heatmap comparing binding interactions of diosmetin and enalaprilat, with residues shown as columns and compounds as rows; dot size and color indicate interaction strength.

rings, indicating its Ca^{2+} antagonistic action. They also confirmed that diosmetin was a different Ca^{2+} channel blocker from verapamil. Furthermore, it stimulated K^{+} channels to partially facilitate diosmetin's vasorelaxant actions.²²

The present study demonstrated that 2K-1C rats had cardiac hypertrophy, as indicated by the elevation of LV wall thickness and cross-sectional areas, and an increase in HW/BW, VW/BW, and LVW/BW. The induction of cardiac morphological changes owing to renal artery obstruction has been supported by several studies.^{49,50} Cardiac hypertrophy is primarily induced by the compensation and adaptation to cardiac high-pressure load.⁵¹ In addition to haemodynamic load, RAS function also influences cardiac hypertrophy.⁵² Therefore, diosmetin treatment may improve cardiac hypertrophy via processes associated with its RAS-inhibitory and anti-hypertensive properties. The alleviation of RAS activation subsequently affected the ACE/Ang II/AT₁R/TGF- β signalling pathway. Similar results have been reported to prove the relationship between ACE/Ang II/AT₁R/TGF- β signalling and the development of cardiac hypertrophy in the experimental models.^{12,53}

2K-1C hypertensive rats also exhibited vascular hypertrophy, as evidenced by increased vascular wall thickness, cross-sectional area, and wall/lumen ratios. This finding corresponds with an earlier analysis showing that vascular hypertrophy is commonly observed in renovascular hypertensive rats.^{54,55} Vascular hypertrophy is an adaptive process that responds to prolonged exposure to haemodynamic changes.^{56,57} In addition, Ang II is a non-haemodynamic component also linked to vascular hypertrophy, which can induce vascular structural changes independent of cardiac effects.⁵⁸ Meanwhile, in cardiac tissue, diosmetin also alleviated vascular hypertrophy by suppressing the ACE/Ang II/AT₁R/TGF- β signalling cascade in vascular tissue in 2K-1C rats. The effect of diosmetin on cardiovascular hypertrophy in the present study was due to the downregulation of AT₁R/TGF- β protein, along with reducing ACE, Ang II, oxidative stress levels, and sympathetic nerve activation. However, this study has some limitations. ACE2 activity and AT2R protein expression are critical factors for supporting the effects of diosmetin related to RAS involvement, but they were not assessed.

Telmisartan was used as the positive control. Telmisartan is a highly selective AT₁R blocker frequently used in hypertension management.⁵⁹ Telmisartan treatment reduces blood pressure, improves vascular function, and suppresses

sympathetic overactivation, RAS overactivity, and cardiovascular hypertrophy in 2K-1C hypertensive rats. This data indicated that the cardiac-aortic effects of telmisartan might be associated with decreasing RAS activation, lowering oxidative stress markers, and downregulating TGF- β protein expression and increasing the levels of Ang-(1-7) and MasR protein expression.^{60,61}

In this study, we investigated the potential of ACE as a molecular target for diosmetin, the aglycone metabolite of diosmin. Diosmetin was selected because it represents the major active form following oral administration of diosmin, which is rapidly hydrolyzed in the gastrointestinal tract.⁶² Molecular docking analysis demonstrated that diosmetin can bind to the catalytic pocket of human ACE (PDB ID: 1UZE), a region defined by a deep, narrow channel that positions the active site approximately midway along the enzyme's length.⁶³ This finding suggests that small molecules like diosmetin are well-suited to access the active site. Our results align with previous reports showing that luteolin, a structural analogue of diosmetin, can also occupy the catalytic pocket and inhibit ACE activity.³⁶ However, whereas luteolin possesses two B-ring hydroxyl groups that interact with Zn²⁺ in a manner similar to the carboxylic acid moiety of enalaprilat, diosmetin contains only one B-ring hydroxyl group because the other is substituted with a methoxy group. This structural difference likely reduces its ability to engage Zn²⁺ effectively, resulting in lower inhibitory potential.³⁶ In contrast, enalaprilat exhibits a stronger binding profile by forming multiple hydrogen bonds and ionic interactions within the catalytic pocket.⁶⁴ Although the binding potency of diosmetin may not match that of enalaprilat, our analysis indicates that it still occupies an important catalytic site, suggesting that it might competitively interfere with ACE function. Further biochemical assays, including crystallographic binding studies, are needed to elucidate these interactions and determine whether this binding profile correlates with experimental inhibitory activity.

Diosmetin is found in various dietary sources, which may facilitate its future advancement as a pharmacological agent owing to its relative safety and availability. Diosmetin demonstrates translational promise as an adjunct therapy for hypertension due to its documented effects. Diosmetin may be particularly advantageous for hypertensive patients who do not achieve blood pressure regulation with antihypertensive medications or who experience unpleasant effects from existing therapies.

Limitations

This study acknowledges some limitations; First, the long-term effects and safety of diosmetin on cardiovascular remodelling or persistent hypertension, particularly in the context of chronic hypertension, have not been established. Second, the lack of results in female rats since sex differences can influence the pathophysiology of hypertension. Third, the molecular docking-based interaction analysis, noting the absence of complementary *in vitro* and *in vivo* validation. Future research should evaluate the effects of diosmetin on chronic hypertension to assess its long-term influence on blood pressure regulation. Translational trials and combination therapy with telmisartan should be explored to elucidate potential additive or synergistic effects. Additionally, future studies should investigate diosmetin's impact on ACE2 activity and AT2R protein expression. *In vitro* and *in vivo* assays are also needed to confirm the *in silico* predictions.

Conclusion

The present study showed that diosmetin exhibits a beneficial effect on haemodynamics and cardiovascular alterations under hypertensive conditions. This effect was mediated by an improvement in endothelial-dependent vasodilation and suppression of sympathetic overactivation, which could be attributed to its ability to inhibit RAS activity, oxidative stress, and AT₁R and TGF- β protein expressions as well as to restore the Ang-(1-7)/MasR pathway. The suppression of RAS was associated with the ACE inhibitor effect of diosmetin, as validated by molecular docking studies. This study suggests that diosmetin may be a beneficial addition in the supplementary therapy of hypertension. It may be utilized in conjunction with telmisartan to provide supplementary advantages for hypertensive patients. The clinical value of diosmetin as a supplementary medication has yet to be elucidated. In terms of comparative effectiveness, telmisartan showed superior efficacy in lowering blood pressure relative to diosmetin; while other parameters such as vascular function, cardiovascular morphology, oxidative stress biomarkers, and RAS parameters showed no significant differences.

Data Sharing Statement

The data for the present study can be assessed by the corresponding author upon reasonable request.

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Disclosure

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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