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Diuretic and Hypotensive Effect of Morelloflavone from *Garcinia dulcis* in Two-Kidneys-One-Clip (2K1C) Hypertensive Rat (Kesan Diuretik dan Hipotensif Moreloflavon daripada *Garcinia dulcis* dalam Dua Buah Pinggang Satu Klip (2K1C) Tikus Hipertensi)

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ABSTRACT

Morelloflavone, a biflavonoid from Southeast Asian folk medical plant *Garcinia dulcis*, possesses powerful antioxidant activity both in vivo and in vitro. We aimed to evaluate the hypotensive and diuretic effect of morelloflavone in two-kidneys-one-clip (2K1C) renovascular hypertensive rats together with its vasorelaxant mechanism. Male Wistar rats (175±4 g) were undergone 2K1C and sham operation (SO) (n=6, each group). Four weeks after the rats were anesthetized and clearance markers (0.5% para-aminohippuric acid and 1% inulin) were given via a jugular vein (1.6 mL/h/100 g BW) to estimate renal blood flow (RBF) and glomerular filtration rate. The arterial blood pressure (AP) was monitored via a carotid artery and urine samples were collected. After equilibration, either morelloflavone or vehicle (DMSO) was given (0.1 mg/kg BW+5 µg/min/kg BW). Baroreflex sensitivity (BRS) ($\Delta HR/\Delta MAP$) was performed by an intravenous injection of 1, 2, 4, 8, 16 and 32 µg/kg BW phenylephrine (PE) or sodium nitroprusside. The PE-precontracted isolated thoracic aortic rings (intact and denuded) relaxation were experimented using organ bath technique by cumulative additions of morelloflavone (10^{-13} - 10^{-5} M) in the presence of specific vasorelaxant inhibitors and expressed as %relaxation from pre-contraction tension. In 2K1C rats, morelloflavone significantly lowered mean AP, increased RBF and increased urine flow rate when compared to vehicle control (138±6 vs. 152±1 mmHg, 3.44±0.49 vs. 2.29±0.25 mL/min/g KW and 42.0±9.4 vs. 18.2±3.9 µL/min/g KW, p<0.05), respectively and also restored the blunt BRS. Nitric oxide signaling pathway triggered by morelloflavone might be responsible for its diuretic and hypotensive effect.

Keywords: Diuretic; hypotensive; *Garcinia dulcis*; morelloflavone; two-kidneys-one-clip

ABSTRAK

Moreloflavon, biflavonoid tumbuhan perubatan tempatan *Garcinia dulcis* dari Asia Tenggara mempunyai aktiviti antioksidan yang kuat secara in vivo dan in vitro. Tujuan penyelidikan ini ialah untuk menilai kesan hipotensif dan diuretik moreloflavon renovascular tikus hipertensi dua buah pinggang satu klip (2K1C) bersama-sama dengan mekanisme vasorelaksan. Tikus jantan Wistar (175±4 g) telah menjalani pembedahan 2K1C dan sham (SO) (n=6, setiap kumpulan). Empat minggu selepas itu, tikus telah dianestetik dan penanda pelepasan (0.5% asid para-aminohipurik dan 1% inulin) diberikan melalui urat jugulum (1.6 mL/jam/100 g BW) untuk menganggar aliran darah ginjal (RBF) dan kadar penurasan glomerulus. Tekanan darah arteri (AP) dipantau melalui arteri karotid dan sampel air kencing diambil. Selepas penyeimbangan, moreloflavon atau wahana (DMSO) telah diberikan (0.1 mg/kg BW+5 µg/min/kg BW). Sensitiviti barorefleks (BRS) ($\Delta HR/\Delta MAP$) telah dijalankan melalui suntikan intravena 1, 2, 4, 8, 16 dan 32 µg/kg BW fenilefrina (PE) atau sodium nitroprusida. PE-praberkuncup yang dipencilkan oleh pengenduran gelang aorta toraks (utuh dan tersuluh) telah dieksperimentasi menggunakan teknik mandian organ dengan penambahan kumulatif moreloflavon (10^{-13} - 10^{-5} M) dengan kehadiran perencatan vasorelaksan tertentu dan dinyatakan sebagai %pengenduran daripada ketegangan pra-pengecutan. Pada tikus 2K1C, moreloflavon dengan ketara menurunkan min AP, meningkatkan RBF serta meningkatkan kadar aliran air kencing berbanding wahana kawalan masing-masing (138±6 vs 152±1 mmHg, 3.44±0.49 vs 2.29±0.25 mL/min/g KW dan 42.0±9.4 vs 18.2±3.9 µL/min/g KW, p<0.05) dan juga memulihkan BRS yang tumpul. Oksida nitrik yang memberi isyarat laluan dicetuskan oleh moreloflavon mungkin bertanggungjawab terhadap kesan diuretik dan hipotensif.

Kata kunci: Diuretik; dua buah pinggang satu-klip; *Garcinia dulcis*; hipotensif; moreloflavon

INTRODUCTION

Garcinia dulcis Kurz, a plant that belongs to the Guttiferae family, is widely distributed in Thailand and other Southeast Asian countries. *G. dulcis* is known as 'maphuut' in Thailand and as 'mundu' in Malaysia and has been used

in folk medicine. Its leaves and seeds are known to treat lymphatitis, parotitis and struma (Kasahara & Henmi 1986) while the stem bark has been used as antiseptic. The fruit juice possesses the properties as an anti-scurvy and expectorant for the relief of cough and sore throat. In

addition, its root extract is also used as an antipyretic and antitoxin agent (Wuttidhamvej 1997).

It has been reported that *G. dulcis* contained at least four groups of chemical compounds including flavonoids (epicatechin, dulcisflavone, morelloflavone), benzophenone (garcinol and camboginol), xanthones (dulcisxanthone and garcinixanthone) (Deachathai et al. 2008, 2006, 2005; Mahabusarakam et al. 2016) and benzophenone-xanthone dimer (garciduols A-C) (Inuma et al. 1996). The amount of chemical constituents extracted from *G. dulcis* depended on the part of the plant specimens and the purified procedure.

Some of isolated phenolic compounds from *G. dulcis* possess various biological activities. As previously reported, dulcisxanthone C-F and dulcinone from the flowers of *G. dulcis* showed a radical scavenging and antibacterial activity (Deachathai et al. 2006), dulcisxanthone G from the seed of *G. dulcis* showed an antibacterial and anti-oxidative activity (Deachathai et al. 2008). Moreover, cambogin, camboginol, dulcisflavone, epicatechin and morelloflavone from the fruits of *G. dulcis* demonstrated the radical trapping and antibacterial activity (Deachathai et al. 2005).

Morelloflavone, a biflavonoid comprising two covalently linked flavones, apigenin and luteolin, occurs in most *Garcinia* species (Ansari et al. 1976; Verbeek et al. 2004). Several biological actions have been reported for morelloflavone. Recently, we reported that in normotensive rats, morelloflavone could induce the dilatation of the isolated thoracic aorta via an endothelial-dependent nitric oxide (NO) signaling pathway (Lamai et al. 2013). Morelloflavone inhibits secretory phospholipase A₂ from human synovial, bee and snake venom (Gil et al. 1997; Pereañez et al. 2014). In animal models it has anti-inflammatory effects, with a potent inhibition of 12-O-tetradecanoylphorbol 13-acetate-induced ear inflammation in mice after topical administration (Gil et al. 1997). Morelloflavone inhibits vascular smooth muscle cell migration, invasion and lamellipodium formation in culture through activation of multiple migration-related kinases, including focal adhesion kinase (FAK), Src, extracellular signal-regulated kinase (ERK) and RhoA (Pinkaw et al. 2009). The inhibition of RhoA and ERK pathways is also observed in vascular endothelial growth factor (VEGF)-stimulated human umbilical cord endothelial cells (Pang et al. 2009). Furthermore, morelloflavone inhibits neointimal proliferation in a mouse model of postangioplasty restenosis (Pinkaw et al. 2009). Morelloflavone inhibits HMG-CoA reductase leading to a decrease in *de novo* cholesterol synthesis (Tuansulong et al. 2011). Oral morelloflavone therapy for 8 months significantly reduced the atherosclerotic areas of the mouse aortae (a 26% reduction), without changing plasma lipid profiles in *Ldlr*^{-/-} *Apobec1*^{-/-} mice (Pinkaw et al. 2012). Despite these reports of various effects of morelloflavone on vascular and inflammatory functions, the effect on cardiovascular and renal functions in a model of hypertension with inflammatory involvement, the two-kidneys-one-clip rat model (2K1C), has not been reported.

The 2K1C model involves unilateral stenosis of the renal artery leading to a permanent reduction in renal perfusion pressure and renal blood flow (RBF) in one kidney (Goldblatt et al. 1934). The resulting hypertension is dependent upon activation of the renin angiotensin system (RAS), which plays an important role in the control of cardiovascular homeostasis affecting both arterial blood pressure (AP) and fluid volume. Angiotensin II, the main effector molecule of the RAS plays a pivotal role in this model by increasing in total peripheral resistance (TPR) and AP (Navar et al. 1998). The 2K1C model also shows impaired endothelial-dependent vasodilatation (Callera et al. 2000). Acute treatment with flavonoids, quercetin and ascorbic acid, improves endothelium-dependent vasodilatation in 2K1C rats (Choi et al. 2016).

Renal function is also altered in 2K1C model, stenosis of one renal artery results in an immediate fall in RBF and glomerular filtration rate (GFR) to that kidney. Three weeks after renal stenosis, the stenotic kidney shows reduced RBF and GFR, whereas the contralateral kidney has a tendency toward reduced GFR in spite of unchanged RBF (Oliveira-Sales et al. 2016). However, RBF and GFR of the non-clipped kidney are similar to that found in normotensive controls by four weeks despite the higher AP, renal vascular resistance (RVR) and angiotensin II level (Anderson et al. 1985; Martinez-Maldonado 1994).

The baroreflex is one of the body's homeostatic mechanisms to modulate blood pressure. Baroreflex sensitivity (BRS) is impaired in animals (Gao et al. 2002) and patients (Maliszewska-Scislo et al. 2008) with renovascular hypertension. Nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase seems to play an important role in the blunted BRS in the 2K1C model, through increased reactive oxygen species (ROS), mainly superoxide anion, production (Botelho-Ono et al. 2011). Furthermore, the effect of endothelial modulation of endothelium-derived NO is impaired in 2K1C hypertension and the responsible mechanism is related to an increase in production of superoxide anions by NADH/NADPH oxidase (Choi et al. 2014).

Against this background of increased ROS signaling and impaired NO function in 2K1C rats, this study aimed to investigate the diuretic and hypotensive effects of morelloflavone in anesthetized normotensive and 2K1C hypertensive rats. The vasorelaxant signaling mechanisms of morelloflavone action were also investigated in isolated thoracic aorta of both 2K1C and SO rats using specific inhibitors.

MATERIALS AND METHODS

EXTRACTION OF MORELLOFLAVONE FROM *G. DULCIS* FRUITS

The fresh ripe fruits of *G. dulcis* were collected from Songkhla province, Thailand. The voucher specimen has been deposited at the Herbarium of Prince of Songkla University, Thailand. The fruits were washed and chopped

into small pieces and then immersed in acetone for 5 days at room temperature. Acetone was removed by evaporation to give a liquid extract that was partitioned with hexane followed by ethyl acetate to give a solid extract. Then, the solid was further fractionated by dissolving in dichloromethane (CH_2Cl_2). The CH_2Cl_2 soluble fraction was further fractionated by column chromatography to give morelloflavone as previously described by Deachathai et al. (2005). Morelloflavone was first isolated in 1976 by Ansari et al. and its structure was illustrated in Figure 1.

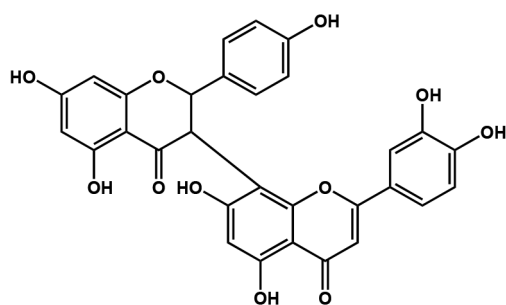


FIGURE 1. Chemical structure of morelloflavone purified from *Garcinia dulcis* fruits, a biflavonoid comprising two covalently linked flavones, apigenin and luteolin (Molecular formula $\text{C}_{30}\text{H}_{20}\text{O}_{11}$, Molecular weight 556) (Ansari et al. 1976)

ANIMALS AND EXPERIMENTAL DESIGN

Male Wistar rats (body weight 175 ± 4 g, $n=84$) were obtained from the Southern Laboratory Animal Facility (Prince of Songkla University, Thailand). Rats were housed under control conditions (temperature $23\text{--}24^\circ\text{C}$; humidity 50-55%; lighting 0600-1800 h), fed a laboratory diet containing 34.2 mmol sodium chloride/kg dry weight food and were allowed free access to reverse osmosis water. All experiments were approved by the Prince of Songkla University Animal Ethics Committee (Reference No. 31/2014).

The study consisted of *in vivo* and *in vitro* parts. The *in vivo* part ($n=24$) consisted of four groups of rats including vehicle-treated sham operation (SO), morelloflavone-treated SO, vehicle-treated 2K1C and morelloflavone-treated 2K1C group ($n=6$, ea). For *in vitro* part ($n=60$), the concentration-response of morelloflavone in isolated aortic rings from 2K1C and SO rats was examined in the absence ($n=6$, ea) and in the presence of the four specific inhibitors ($n=6$, ea).

ESTABLISHMENT OF 2K1C HYPERTENSIVE RAT

The rats were anaesthetized with a single dose of pentobarbital sodium (50 mg/kg BW i.p.; CEVA Santé Animal, Brussels, Belgium). Only the deep sedated animals were gone through the following surgical protocol; the left kidney was exposed through a 1 cm retroperitoneal incision, the left renal artery was then exposed and cleared

from surrounding connective tissues and a U-shaped silver clip with a 0.20 mm gap was placed around it close to the junction with the abdominal aorta. The free edges of the clip were compressed firmly to prevent dislodging. At the end of surgery, the muscle and skin layer were sutured separately with catgut and silk No. 4/0, respectively. The SO included the entire surgery with the exception of renal artery clipping. At the end of the surgery, all animals received a single dose of ampicillin (50 mg/kg BW, i.m.) injection and were allowed to recover in separate cages for 2-3 h under an angle poise lamp and remained untouched for 4 weeks afterward in order to develop hypertension.

EXPERIMENTAL PROTOCOL FOR THE STUDY OF RENAL CLEARANCE AND BARORECEPTOR REFLEX

Four weeks after the operation, renal clearance and BRS were studied in one cohort of animals ($n=6$). The rats were anesthetized with pentobarbital sodium (60 mg/kg BW i.p.; an additional dose was given when necessary) and placed on a thermostatically-controlled heated table to maintain body temperature at 37°C . A tracheotomy was performed and the left carotid artery was cannulated, using polyethylene tube (PE-50) filled with heparinized 0.9% NaCl and connected to a pressure transducer, coupled to a PowerLab system (ADInstruments, Colorado Springs, CO, USA), to measure AP and heart rate (HR) and for blood sampling. The right jugular vein was cannulated, using PE-50, and infused with clearance markers containing 1% inulin (Sigma Chemical Co., St. Louis, MO, USA) and 0.5% p-amino-hippuric acid (PAH) (Sigma Chemical Co., St. Louis, MO, USA) dissolved in 0.9% NaCl at a rate of 1.6 mL/h/100g BW. Via a suprapubic midline incision, the left and right ureters were cannulated, using PE-10 connected to PE-50 for urine sample collection. After 45 min equilibration, morelloflavone dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA) was given via the jugular vein as a bolus injection (0.1 mg/kg BW; volume of injection was 0.05 mL/100g BW) and then followed by continuous infusion at the rate of $5 \mu\text{g}/\text{min}/\text{kg}$ BW along with the clearance markers. This experimental dose of morelloflavone was chosen based on the minimal effective dose of a specific angiotensin II receptor antagonist, candesartan (Hiranyachattada et al. 2005). The same amount of DMSO was given as vehicle in both SO and 2K1C groups. To determine the renal clearance, three consecutive 20 min urine collections were made during the 60 min of experimental period. An arterial blood sample (400 μL) was taken at the mid-point of the first and third urine collecting period. A small amount of the blood sample was used to determine the hematocrit by the microcapillary method and the remainder was centrifuged and the plasma was collected for determination of the concentrations of inulin and PAH. Blood cells were re-suspended in 200 μL 0.9% NaCl and returned to the animal via the right jugular vein.

Urine flow rate was determined gravimetrically by collecting urine into pre-weighed tubes and assuming a

density of 1 g/mL. The PAH and inulin concentrations, either in plasma or urine samples, were determined by a spectrophotometric method according to Smith et al. (1954) and Davidson and Sackner (1963), respectively. Plasma and urine osmolality PO_{sm} and UO_{sm} were measured by using Micro-Osmometer (Fiske Model 210, USA) and then osmolar and negative free water clearance (CO_{sm} and TCH_2O) were calculated.

The BRS determination was performed after the 60 min of renal clearance study by measuring HR and AP before and after acute injection of the two vasoactive drugs, phenylephrine (PE) (Sigma Chemical Co., St. Louis, MO, USA), a specific α_1 receptor agonist and sodium nitroprusside (SNP) (Sigma Chemical Co., St. Louis, MO, USA), a nitric oxide donor, at the same doses of 1, 2, 4, 8, 16 and 32 $\mu\text{g}/\text{kg}$ BW.

At the end of experiment, rats were killed by intravenous injection of saturated $MgSO_4$. Both kidneys were removed, decapsulated, dried on blotting paper and weighed. The heart was also removed and weighed after carefully clearing away blood vessels, fat and connective tissues. The organ weight to total body weight ratio was calculated.

PREPARATION OF ISOLATED THORACIC AORTIC RINGS

After 4 weeks, separate groups of 2K1C and SO rats ($n=6$, ea) were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and sacrificed by decapitation. The thoracic aorta was dissected and cut into four ring segments approximately 5 mm in length each (two intact- and two denuded-endothelial rings). The denuded rings were performed by mechanical removal of endothelium. The rings were mounted in 20 mL organ baths containing 37°C Krebs Henseleit solution which was composed of (mM) 118.41 NaCl, 4.6 KCl, 1.12 $MgSO_4 \cdot 7H_2O$, 1.18 KH_2PO_4 , 1.9 $CaCl_2$, 25.0 $NaHCO_3$ and 11.66 D-glucose. The pH of solution was maintained at 7.4 by continuous aeration with 95% O_2 and 5% CO_2 . The resting tension of 1 g was set and the tension changes during the courses of experiment were recorded using force displacement transducer (Model FT03, Grass Instrument Co., Quincy, MA, USA) connected to PowerLab system (ADInstruments, USA). Endothelial function of aortic rings was tested by the addition of 10^{-5} M acetylcholine (ACh) (Sigma Chemical Co., St. Louis, MO, USA) into the 10^{-7} M PE precontracted rings. The 80% relaxation was accepted and considered as an intact endothelium and the disappearance of relaxation was considered denuded endothelium (Molina et al. 1992).

CONCENTRATION-RESPONSE CURVE OF MORELLOFLAVONE

After 45 min equilibration, aortic rings (both intact and denuded endothelium) from 2K1C and SO rats were precontracted by addition of 10^{-7} M PE. When the maximal contraction response developed, the tension was recorded.

Either morelloflavone or vehicle (0.1% DMSO) was added cumulatively, allowing the final concentration to be 10^{-13} - 10^{-5} M. Subsequent concentrations were added after the maximal response by the previous concentration developed and recorded.

EFFECT OF SPECIFIC INHIBITORS ON VASORELAXATION RESPONSE OF MORELLOFLAVONE

After 15 min equilibration, the intact-endothelial aortic rings from 2K1C and SO rats were incubated with each specific inhibitor for 30 min before precontraction with 10^{-7} M PE. The doses of each inhibitor were 10^{-4} M $N\omega$ -Nitro-L-arginine methyl ester (L-NAME) (Sigma Chemical Co., St. Louis, MO, USA), 10^{-6} M indomethacin (Sigma Chemical Co., St. Louis, MO, USA), 10^{-5} M glibenclamide (Sigma Chemical Co., St. Louis, MO, USA) and 10^{-3} M tetraethyl ammonium (TEA) (Sigma Chemical Co., St. Louis, MO, USA). Then, either morelloflavone (10^{-13} - 10^{-8} M) or vehicle (0.1% DMSO) was added cumulatively after the maximal contraction was developed and 5-7 min sustained. Subsequent concentrations were added after the maximal response by the previous concentration developed and recorded.

CALCULATIONS AND STATISTICAL ANALYSES

Inulin, PAH and osmolar clearance were calculated according to clearance equation ($\dot{V} \times UX/PX$), free water clearance (CH_2O) as $\dot{V} - CO_{sm}$ and negative free water clearance (TCH_2O) as $CO_{sm} - \dot{V}$, where \dot{V} = urine flow rate, UX and PX = urine and plasma concentration of either inulin, PAH or urine and plasma osmolality, CO_{sm} = osmolar clearance. Clearance of inulin and PAH were taken as the indices of GFR and effective renal plasma flow (ERPF), respectively. CO_{sm} and TCH_2O were the indices of urinary electrolyte excretion and free water reabsorption, respectively. Mean arterial pressure (MAP) was determined from diastolic blood pressure (DBP) + 1/3 pulse pressure (PP). Renal vascular resistance (RVR) was computed from $\Delta MAP/ERPF$ and expressed as resistance unit (RU). BRS was computed from $\Delta HR/\Delta MAP$. All data were expressed as the mean \pm S.E.M. Comparisons between the mean values were performed with one-way analysis of variance (ANOVA) (GraphPad Prism 5, San Diego, CA, USA) followed by Tukey post hoc test. A $p < 0.05$ was considered to be statistically significant difference.

For *in vitro* study, the degree of vasorelaxation in each experiment were expressed as a %relaxation from PE (10^{-7} M) precontraction tension. All values were expressed as mean \pm S.E.M. Significant difference between the group means was determined using ANOVA followed by Student-Newman Keuls post hoc test or student t-test. The negative logarithm (pD_2) and half maximal effective concentration (EC_{50}) values were calculated using GraphPad Prism 5 (San Diego, CA, USA). Statistical significance of the mean differences were accepted when $p < 0.05$.

RESULTS

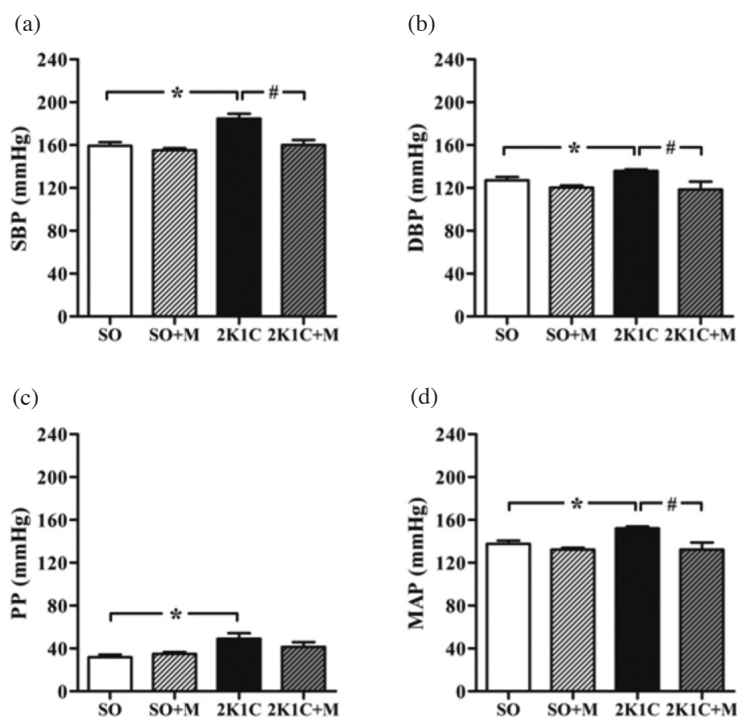
EFFECTS OF MORELLOFLAVONE ON ARTERIAL BLOOD PRESSURE AND HEART RATE

As shown in Figure 2(a)-2(d), four weeks after experimental renal stenosis, the systolic blood pressure (SBP), DBP, PP and MAP in 2K1C rats were significantly higher than in those of SO rats (SBP; 185 ± 4 vs. 155 ± 2 , DBP; 136 ± 1 vs. 125 ± 4 , PP; 49 ± 4 vs. 30 ± 2 and MAP; 152 ± 1 vs. 135 ± 3 mmHg, respectively, $p < 0.05$). The resting HR of 2K1C group was not significantly different from SO (208 ± 6 vs. 213 ± 3 bpm). When morelloflavone was given in 2K1C rats, the SBP, DBP and MAP decreased significantly when compared to vehicle-treated 2K1C rats (SBP; 165 ± 4 vs. 185 ± 4 , DBP; 126 ± 5 vs. 136 ± 1 and MAP; 138 ± 6 vs. 152 ± 1 mmHg, respectively, $p < 0.05$) whereas the PP and HR did not alter statistically (PP; 41 ± 4 vs. 49 ± 4 mmHg and HR; 207 ± 5 vs. 208 ± 6 bpm). The treatment of either morelloflavone or its vehicle in SO rats showed no significant alterations in SBP (155 ± 2 vs. 155 ± 2 mmHg), DBP (121 ± 2 vs. 125 ± 4 mmHg), PP (35 ± 2 vs. 30 ± 2 mmHg), MAP (132 ± 2 vs. 135 ± 3 mmHg) and HR (203 ± 5 vs. 213 ± 3 bpm), respectively. The significant higher AP observed in 2K1C than SO rats suggested the successful development of hypertensive model in this study. Morelloflavone treatment lowered AP observed in 2K1C rats towards the level in SO.

EFFECTS OF MORELLOFLAVONE ON RENAL FUNCTIONS

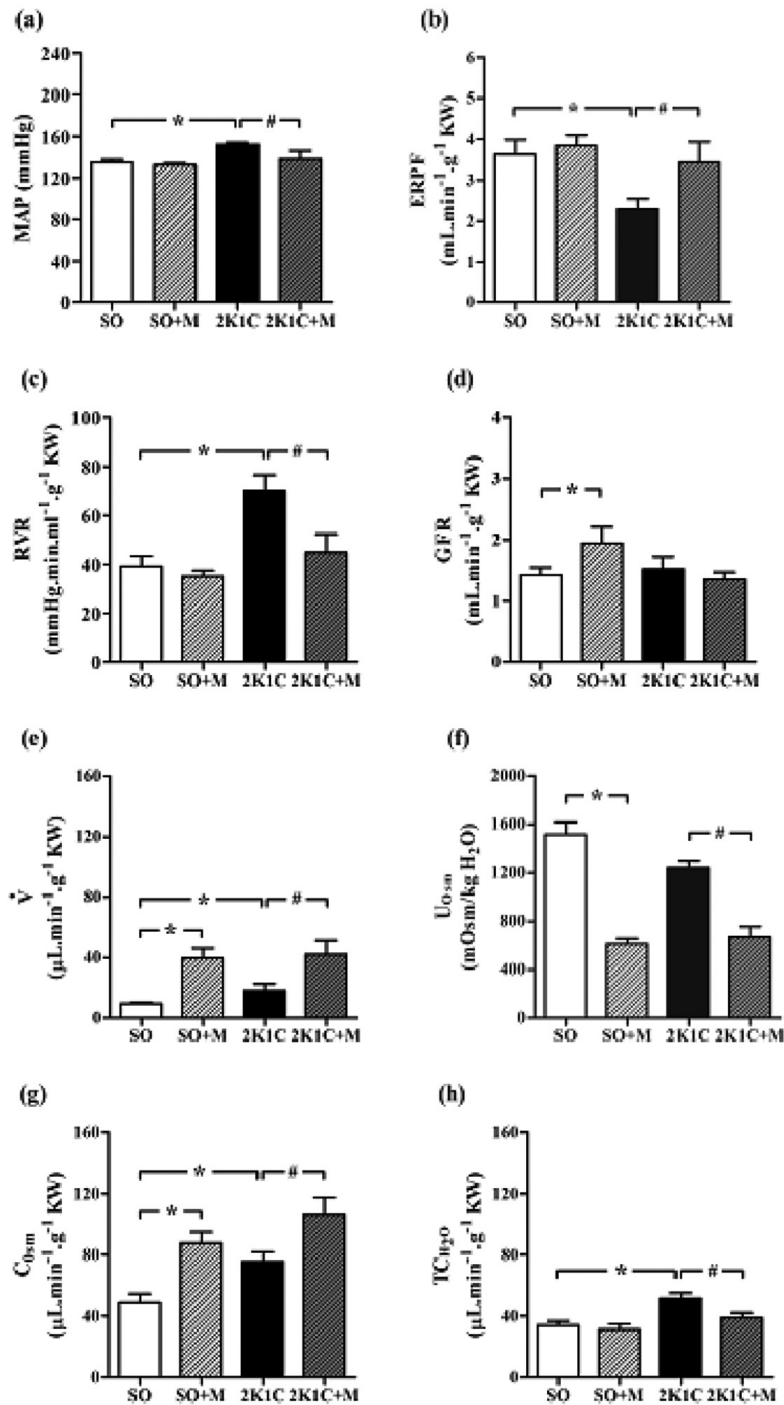
As shown in Figure 3(a)-3(h), 2K1C rats had significantly higher renal vascular resistance (RVR), urine flow rate (\dot{V}), osmolar clearance (CO_{sm}) and negative free water clearance (TCH_2O) when compared to SO rats (RVR; 70 ± 6 vs. 39 ± 4 RU; ; 18.2 ± 3.9 vs. 9.6 ± 0.9 $\mu\text{L}/\text{min}/\text{g}$ KW, CO_{sm} ; 75.6 ± 5.8 vs. 52.1 ± 3.8 $\mu\text{L}/\text{min}/\text{g}$ KW and TCH_2O ; 56.2 ± 5.4 vs. 41.0 ± 3.6 $\mu\text{L}/\text{min}/\text{g}$ KW, respectively, $p < 0.05$) but had a significantly lower ERPF (2.29 ± 0.25 vs. 3.63 ± 0.36 mL/min/g KW, $p < 0.05$). However, there was no significant difference of GFR, urine and plasma osmolality (U_{Osm} and P_{Osm}) between 2K1C and SO rats (GFR; 1.48 ± 0.16 vs. 1.43 ± 0.12 mL/min/g KW, U_{Osm} ; 1240 ± 49 vs. 1425 ± 46 mOsm/kg H_2O and P_{Osm} ; 330 ± 5 vs. 326 ± 3 mOsm/kg H_2O , respectively).

When morelloflavone was given in 2K1C rats, a significant decrease in RVR, U_{Osm} and TCH_2O were observed when compared to vehicle-treated 2K1C rats (RVR; 45 ± 6 vs. 70 ± 6 RU, U_{Osm} 670 ± 84 vs. 1240 ± 49 mOsm/kg H_2O and TCH_2O ; 40.4 ± 3.8 vs. 56.2 ± 5.4 $\mu\text{L}/\text{min}/\text{g}$ KW, respectively, $p < 0.05$). However, the ERPF, \dot{V} and CO_{sm} were found to be significantly higher (ERPF; 3.44 ± 0.49 vs. 2.29 ± 0.25 mL/min/g KW, \dot{V} ; 42.0 ± 9.4 vs. 18.2 ± 3.9 $\mu\text{L}/\text{min}/\text{g}$ KW and CO_{sm} ; 106.3 ± 11.4 vs. 75.6 ± 5.8 $\mu\text{L}/\text{min}/\text{g}$ KW, respectively, $p < 0.05$). In addition, GFR and P_{Osm} were not significantly different between the two groups (GFR; 1.48 ± 0.16 vs. 1.35 ± 0.12 mL/min/g KW and P_{Osm} ; 317 ± 4 vs. 330 ± 4 mOsm/kg H_2O , respectively).



Values are mean \pm S.E.M.*
$p < 0.05$ compare with SO and 2K1C group, respectively (one-way ANOVA)

FIGURE 2. Effects of morelloflavone (M) 0.1 mg/kg BW + 5 $\mu\text{g}/\text{min}/\text{kg}$ BW on systolic blood pressure (SBP; a), diastolic blood pressure (DBP; b), pulse pressure (PP; c) and mean arterial blood pressure (MAP; d) in 2-kidneys-1-clip (2K1C) and sham operative (SO) group during clearance study



Values are mean ± S.E.M.*
#*p*<0.05 compare with SO and 2K1C group, respectively (one-way ANOVA)

FIGURE 3. Effects of morelloflavone (M) (0.1mg/kg BW bolus + 5 μg/min/kg BW) on mean arterial blood pressure (MAP; a), effective renal plasma flow (ERPF; b), renal vascular resistance (RVR; c), glomerular filtration rate (GFR; d), urine flow rate (\dot{V} ; e), urine osmolality (U_{Osm}; f), osmolar clearance (C_{Osm}; g) and negative free water clearance (TCH₂O; h), in 2-kidneys-1-clip (2K1C) and sham operative (SO) group during clearance study

The administration of morelloflavone in SO rats resulted in a significant increase in GFR, \dot{V} and C_{Osm} but a decrease in U_{Osm} when compared to vehicle-treated SO rats (GFR; 1.72±0.14 vs. 1.43±0.12 mL/min/g KW, \dot{V} ; 44.7±4.3 vs. 9.6±0.9 μL/min/g KW, C_{Osm}; 87.5±7.2 vs.

52.8±5.6 μL/min/g KW and U_{Osm}; 613±41 vs. 1425±46 mOsm/kg H₂O, respectively, *p*<0.05) while the ERPF, RVR, P_{Osm} and TCH₂O remained unaltered (ERPF; 3.96±0.28 vs. 3.63±0.36 mL/min/g KW, RVR; 35±2 vs. 39±4 RU, P_{Osm}; 321±2 vs. 326±3 mOsm/kg H₂O and TCH₂O; 31.7±3.8 vs.

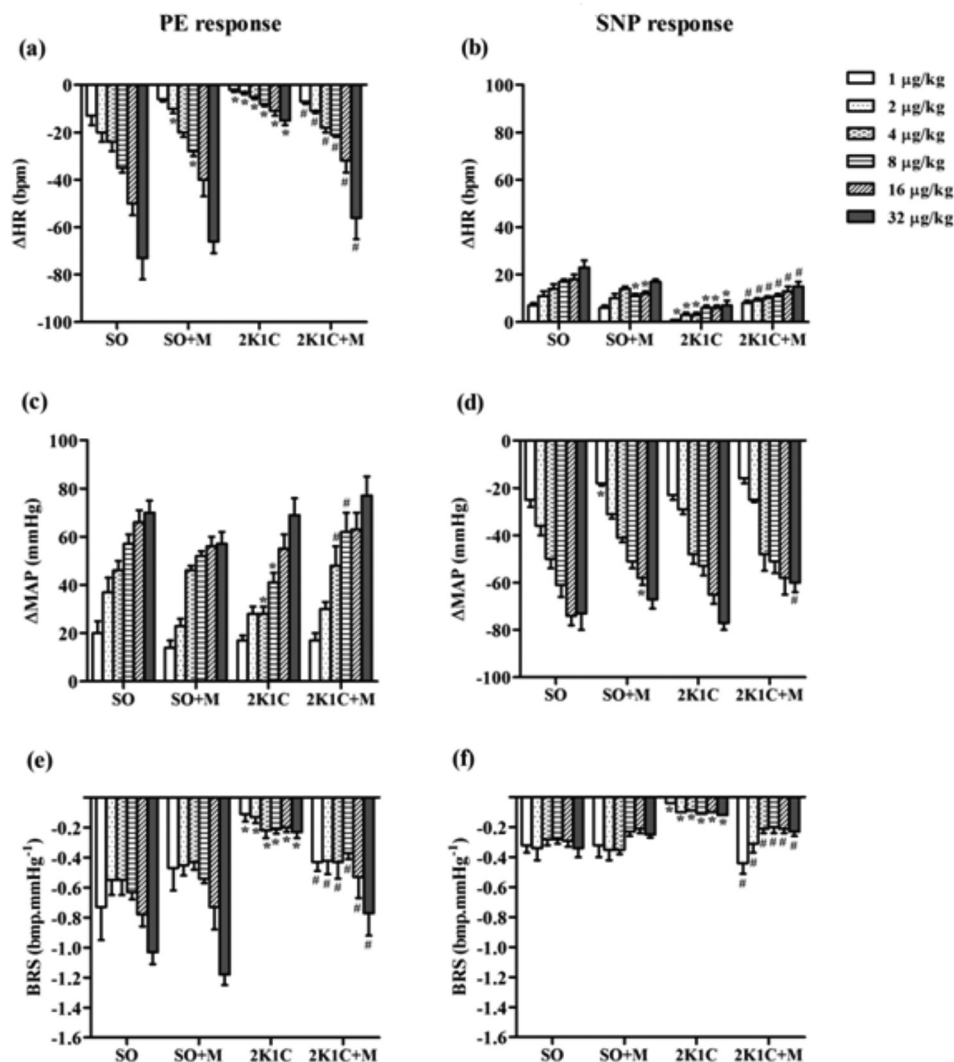
40.0±3.4 $\mu\text{L}/\text{min}/\text{g}$ KW, respectively). All the stated data suggested that the decreased RBF of 2K1C rats was due to the renal vasoconstriction. Renal tubular water and electrolytes excretion of 2K1C rats were also impaired. Morelloflavone treatment can correct the impairment of both RBF and renal electrolytes excretion in 2K1C rats. The diuretic effect of morelloflavone was also observed in SO rats.

EFFECTS OF MORELLOFLAVONE ON BAROREFLEX SENSITIVITY (BRS)

As shown in Figure 4(a) and 4(b), the BRS responses to the six doses of PE (1, 2, 4, 8, 16 and 32 $\mu\text{g}/\text{kg}$ BW) intravenous injection in 2K1C were significantly lowered when compared to those of SO rats (-0.11±0.05 vs. -0.73±0.22, -0.13±0.04 vs. -0.55±0.10, -0.22±0.05 vs.

-0.55±0.10, -0.21±0.03 vs. -0.63±0.05, -0.20±0.03 vs. -0.78±0.08 and -0.23±0.04 vs. -1.03±0.08 bpm/mmHg, respectively, $p<0.05$). There was no significant difference in respective BRS to PE between SO with morelloflavone-treated and vehicle-treated rats. Morelloflavone treatment significantly resulted in a higher BRS in 2K1C in comparison to vehicle-treated rats at the respective doses of PE injection (-0.43±0.06 vs. -0.11±0.05, -0.42±0.09 vs. -0.13±0.04, -0.43±0.11 vs. -0.22±0.05, -0.37±0.04 vs. -0.21±0.03, -0.53±0.14 vs. -0.20±0.03 and -0.77±0.15 vs. -0.23±0.04 bpm/mmHg, respectively, $p<0.05$).

The BRS to the six doses of SNP (1, 2, 4, 8, 16 and 32 $\mu\text{g}/\text{kg}$ BW) intravenous injection in 2K1C rats were significantly lowered when compared to those of SO rats (-0.04±0.02 vs. -0.32±0.05, -0.10±0.04 vs. -0.34±0.08, -0.09±0.04 vs. -0.28±0.04, -0.11±0.03 vs.



Values are mean ± S.E.M.*

* $p<0.05$ compared with SO and 2K1C group at the respective concentrations of phenylephrine (PE) or sodium nitroprusside (SNP) (one-way ANOVA)

FIGURE 4. Baroreflex sensitivity (BRS) in response to either phenylephrine (PE; left panel) or sodium nitroprusside (SNP; right panel) in 2-kidneys-1-clip (2K1C) and sham operative (SO) group during treatment with morelloflavone (M) (0.1mg/kg BW + 5 $\mu\text{g}/\text{min}/\text{kg}$ BW). BRS was computed from $\Delta\text{HR}/\Delta\text{MAP}$

-0.28±0.03, -0.10±0.02 vs. -0.29±0.04 and -0.12±0.02 vs. -0.34±0.06 bpm/mmHg, respectively, $p<0.05$). There was no significant difference in BRS between SO with merelloflavone-treated and vehicle-treated rats at the respective doses of SNP.

Morelloflavone treatment significantly resulted a higher BRS in 2K1C in comparison to vehicle-treated rats at the respective doses of SNP injection (-0.44±0.07 vs -0.04±0.02, -0.31±0.06 vs. -0.10±0.04, -0.21±0.03 vs. -0.09±0.04, -0.20±0.04 vs. -0.11±0.03, -0.21±0.03 vs. -0.10±0.02 and -0.23±0.03 vs. -0.12±0.02 bpm/mmHg, respectively, $p<0.05$). The above data suggested that the BRS were blunted in 2K1C rats and morelloflavone treatment can restore this BRS impairment.

CHANGES IN KIDNEY WEIGHT, CARDIAC MASS AND BODY WEIGHT

As shown in Table 1, the left clipped kidneys of vehicle-treated 2K1C groups atrophied while the right unclipped kidneys hypertrophied when compared with the respective ipsilateral kidneys of vehicle-treated SO groups (left KW/BW; 0.07±0.01 vs. 0.27±0.01% and right KW/BW; 0.39±0.02 vs. 0.30±0.01%, respectively, $p<0.05$). Cardiac mass/BW of vehicle-treated 2K1C significantly increased in comparison to that of vehicle-treated SO rats (0.32±0.02 vs. 0.27±0.01%, respectively, $p<0.05$). Other parameters including the pre- and post-body weight, body weight changes and hematocrit values measuring during clearance study were not significantly different between among those four groups of rats.

VASORELAXANT EFFECTS OF MORELLOFLAVONE IN ISOLATED THORACIC AORTIC RINGS

As shown in Figure 5(a), the addition of the cumulative doses of morelloflavone (10^{-13} - 10^{-5} M) significantly relaxed the PE-precontracted endothelial intact aortic rings in a concentration-dependent manner with the $pD_2 = 6.99 \pm 0.91$ ($EC_{50} = 102.8$ nM) of 2K1C and the $pD_2 = 9.92 \pm 0.86$ ($EC_{50} =$

0.52 nM) of SO rats ($p<0.05$). Denudation of the functional endothelium completely abolished morelloflavone-induced vasorelaxation in both 2K1C and SO as shown in Figure 5(b). These data indicated the vasorelaxation effect of morelloflavone which might be endothelium dependent.

EFFECT OF L-NAME ON MORELLOFLAVONE-INDUCED VASORELAXATION

Pretreatment of the endothelium-intact aortic rings with 10^{-4} M L-NAME significantly and completely abolished (10^{-12} - 10^{-8} M) morelloflavone-induced vasorelaxation in both SO and 2K1C rats as shown in Figure 6(a) and 6(b). The data suggested that vasorelaxant effect of morelloflavone might involve in endothelial NO signaling pathway.

EFFECTS OF INDOMETHACIN, GLIBENCAMIDE AND TEA ON MORELLOFLAVONE-INDUCED VASORELAXATION

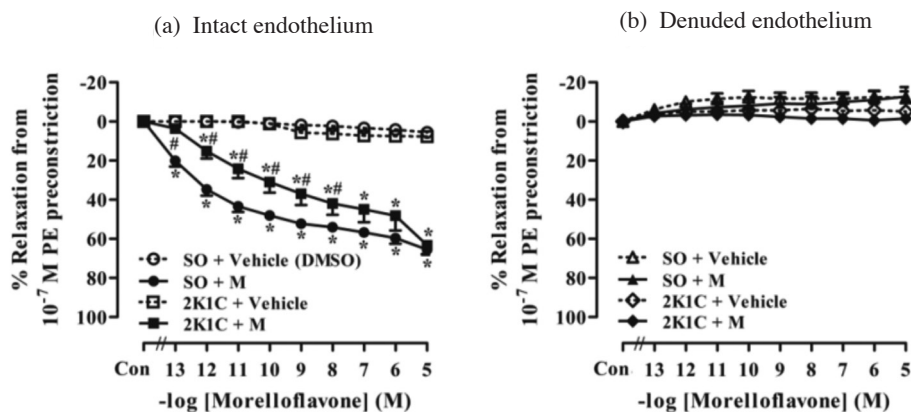
As shown in Figure 6(d) and 6(h), the %relaxation of aortic rings from SO rats in response to the addition of morelloflavone (10^{-13} and 10^{-12} M) in the presence of 10^{-6} M indomethacin and 10^{-5} M glibencamide were found to be significantly decreased in comparison to in the absence of indomethacin (10^{-13} M morelloflavone; 3.3±0.9 vs. 20.1±3.1% and 10^{-12} M; 17.7±4.1 vs. 34.8±3.4 %, respectively, $p<0.05$) and glibenclamide (10^{-13} M morelloflavone; 5.9±1.4 vs. 20.0±4.2 % and 10^{-12} M; 19.9±4.1 vs. 33.6±3.6 %, respectively, $p<0.05$). The %relaxation in response to morelloflavone (10^{-13} - 10^{-11} M) in the presence of 10^{-3} M TEA were found to be significantly decreased in comparison to in the absence of this inhibitors (10^{-13} M morelloflavone; 2.2±1.3 vs. 20.0±4.2, 10^{-12} M; 12.4±4.5 vs. 33.6±3.6 and 10^{-11} M; 27.4±5.8 vs. 45.2±4.6%, respectively, $p<0.05$) as shown in Figure 6(f). The effects of these three inhibitors at the lower doses of morelloflavone-induced vasorelaxation occurred only in SO but not 2K1C (Figure 6(c), 6(e) and 6(g)), suggested that the mechanism of morelloflavone actions may involve PGI_2 , K_{ATP} and $K_{Ca^{2+}}$ signaling pathways.

TABLE 1. Comparison of body weight (BW), left and right kidney weight (KW), cardiac mass and hematocrit between sham operation (SO) and 2-kidneys-1-clip (2K1C) rats which treated with either morelloflavone (M) or vehicle (V)

	SO+V	SO+M	2K1C+V	2K1C+M
Number of rats	6	6	6	6
Pre-BW (g)	206 ± 4	202 ± 3	194 ± 9	186 ± 9
Post-BW (g)	388 ± 3	383 ± 10	379 ± 20	358 ± 13
ΔBW (g)	182 ± 6	181 ± 10	185 ± 19	172 ± 19
Left KW (g)	1.05 ± 0.02	0.99 ± 0.02	0.28 ± 0.07*	0.23 ± 0.02*
Left KW/BW (%)	0.27 ± 0.01	0.26 ± 0.01	0.07 ± 0.01*	0.06 ± 0.01*
Right KW (g)	1.18 ± 0.02	1.05 ± 0.03	1.46 ± 0.07*	1.25 ± 0.03*
Right KW/BW (%)	0.30 ± 0.01	0.27 ± 0.01	0.39 ± 0.02*	0.35 ± 0.01*
Cardiac mass (g)	1.06 ± 0.02	1.00 ± 0.03	1.23 ± 0.12*	1.22 ± 0.02*
Cardiac mass/BW (%)	0.27 ± 0.01	0.26 ± 0.01	0.32 ± 0.02*	0.34 ± 0.01*
Hematocrit (%)	49.2 ± 0.9	52.0 ± 0.4	51.6 ± 1.3	51.6 ± 0.8

Data are mean ± S.E.M.

* $p<0.05$ compare with respective SO group (Student t-test)



Values are mean \pm S.E.M of the percentage relaxation from 10^{-7} M phenylephrine (PE) pre-contraction*
p <0.05 compare with respective vehicle groups and SO+M group, respectively (one-way ANOVA)

FIGURE 5. Effects of morelloflavone (M) or vehicle (DMSO) on vasorelaxation of intact endothelium (a) or denuded (b) thoracic aorta from 2-kidneys-1-clip (2K1C) or sham operative (SO) group

DISCUSSION

Four weeks after experimental renal artery stenosis, SBP, DBP, PP and MAP of 2K1C measuring under pentobarbitone sodium anesthetization via left carotid artery were significantly higher than those of SO. The results suggested the successful development of hypertensive model in this study. This was also supported by the significant increase in the cardiac mass and the atrophy of the clipped left kidney with the hypertrophy of the non-clipped right kidney of 2K1C when compared to SO rats (Table 1). One of the important pathological mechanisms in hypertension development involves the abnormality of RAS in which the high circulating angiotensin II directly binds to the receptor at vascular smooth muscle and then increases the total peripheral resistance and raises blood pressure observed in both awoken and under anesthesia rats (Goldblatt et al. 1934; Murphy et al. 1984; Navar et al. 1998). The higher renal vascular resistance seen in 2K1C than in SO (Figure 3(c)), in part, positively supported an increase in total vascular resistance by increased angiotensin II level. Moreover, the role of oxidative stress in generation and maintenance of arterial hypertension has been reported in 2K1C model (Oliveira-Sale et al. 2008). It was shown that an increase in circulating angiotensin II can activate the vascular smooth muscle NAD(P)H oxidase which is an important cellular source of ROS (Griendling et al. 1994). In addition, the rise in superoxide level decreases the bioavailability of NO and contributes to the maintenance of elevated peripheral resistance (Cai & Harrison 2000).

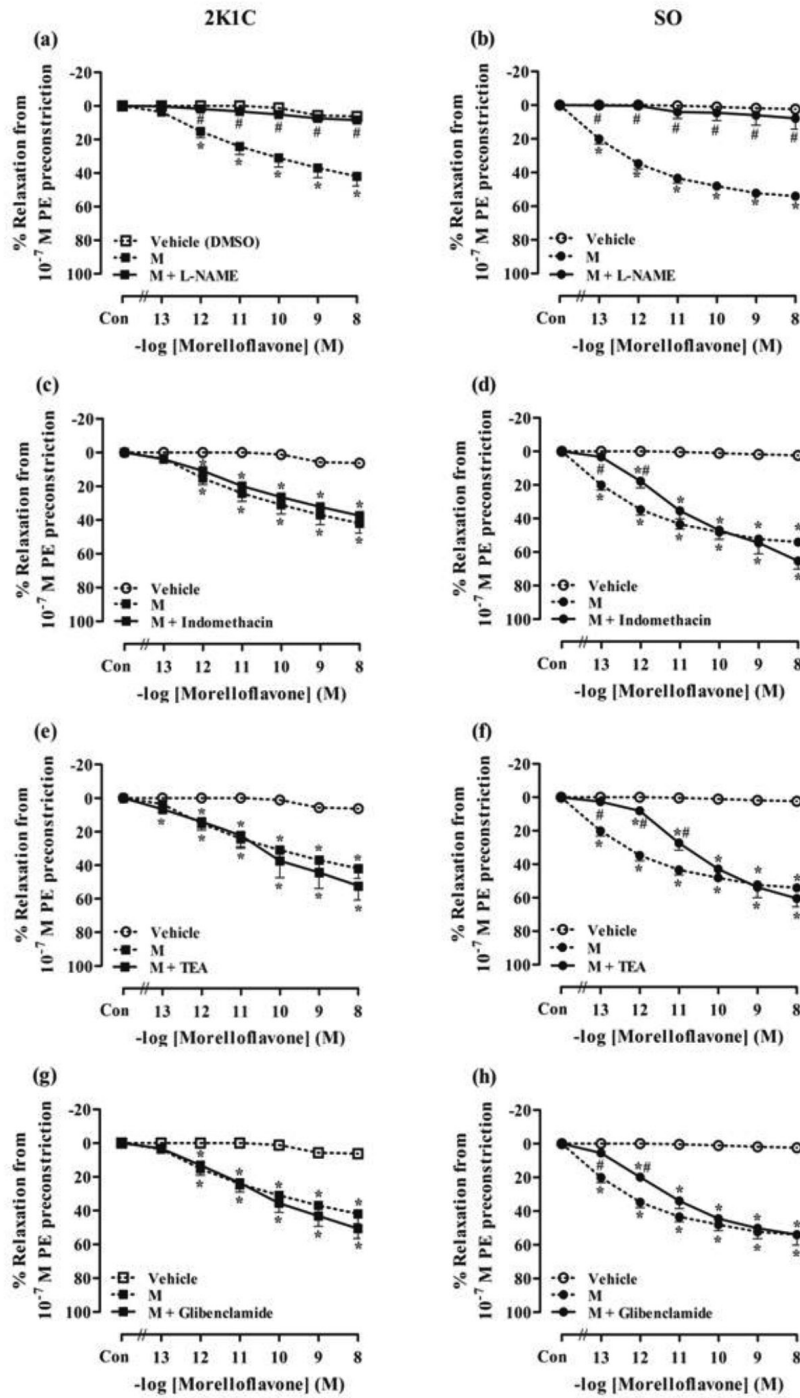
Morelloflavone administration in 2K1C rats in this study significantly lowered SBP, DBP and MAP which was not found in SO rats (Figure 2). These results suggested the pronounced hypotensive effect of morelloflavone in hypertension rather than in normotension. Experiments in isolated thoracic aorta showed the vasorelaxant mechanism of morelloflavone in 2K1C is likely to be endothelium-dependent and involves NO signaling

pathway similar to those of SO rats (Figures 5 and 6(a), 6(b)) even though the more endothelium damage occurred in 2K1C than SO (Figure 5(a) and their EC_{50} values). The other signaling pathways which may contribute to the vasorelaxant mechanism of morelloflavone in normotension included the operation of $K_{Ca^{2+}}$, K_{ATP} channels or endothelial derived hyperpolarizing factor (EDHF) pathway which were unlikely to occur in 2K1C hypertension (Figure 6(c)-6(h)).

Thus, the marked hypotensive effect of morelloflavone seen in 2K1C rats may be due to its both antioxidant and NO-dependent vasorelaxant property. The reason that this hypotensive effect was not obviously seen in SO rats may be the well regulation of baroreceptor reflex in response to the chosen dose of morelloflavone (0.1 mg/kg+5 μ g/kg/min). This dose was the minimal hypotensive dose comparable to candesartan, an angiotensin II receptor blocker previously reported (Hiranyachattada et al. 2005). At this minimal hypotensive effective dose of morelloflavone selected in this study, taken together with the more impaired BRS of 2K1C than SO, the pronounced reduction in arterial blood pressure was then observed.

The BRS responses to PE and SNP in 2K1C were less than SO rats suggested the blunt BRS (Figure 4) and morelloflavone could restore these impaired BRS. The responsible mechanism may occur via its antioxidant property which enhanced NO bioavailability since it is reported that an acute infusion antioxidant such as vitamin C or apocyanin, a NADPH oxidase inhibitor, can restore BRS in 2K1C rats to the values that resembles to those of normotensive rats (Botelho-Ono et al. 2011). Acute superoxide scavenging is also reported to restore the depressed BRS in 2K1C hypertensive rats (Botelho-Ono et al. 2011).

Beside the marked hypotensive effect of morelloflavone in 2K1C rats, its diuretic effect was also investigated. While there was no change in GFR of 2K1C when compared to SO rats, RVR, \dot{V} , CO_{sm} and TCH_{2O}



Values are mean \pm S.E.M.*
 # $p < 0.05$ compare with vehicle and morelloflavone (M) group, respectively (one-way ANOVA)

FIGURE 6. Effects of morelloflavone (M) on vasorelaxation of intact endothelium thoracic aorta from 2-kidneys-1-clip (2K1C; left panels) and sham operation (SO; right panels) group in the presence of specific inhibitors; 10^{-4} M L-NAME (a, b), 10^{-6} M indomethacin (c, d), 10^{-3} M TEA (e, f) and 10^{-5} M glibenclamide (g, h)

were significantly higher. These findings are consistent with previous reports in which the GFR of 2K1C rats is similar to normotensive controls within 4 weeks after the renal artery clipping despite the increasing in AP, RVR and angiotensin II level (Anderson et al. 1985;

Martinez-Maldonado1991). The higher urine flow in 2K1C may be due to an abnormal tubular electrolyte and water reabsorption in response to high circulating level of angiotensin II or NO similar to experimented in normotension. In normotensive rats, angiotensin II exerts

biphasic effect in which low dose (10^{-12} - 10^{-9} M) when applying to either luminal or peritubular side stimulates renal tubular fluid absorption while the higher dose (10^{-7} - 10^{-6} M) causes inhibition (Harris & Young 1997). Moreover, addition of SNP, a NO donor, inhibits renal proximal fluid reabsorption (Eitle et al. 1998). Thus, the inhibition of tubular electrolytes and water reabsorption due to the higher levels of angiotensin II and perhaps NO may contribute to the high urine flow rate and high osmolar clearance of 2K1C.

The diuretic effect of morelloflavone was not only observed in normotensive SO but also in hypertensive 2K1C with the similar degree, however, the mechanisms of diuretic action might be different in some extent. After morelloflavone administration in SO rats, GFR, urine flow rate and osmolar clearance increased significantly when compared to the vehicle-treated SO rats while other renal function parameters remained unaltered. These findings suggested the diuretic effect of morelloflavone in normotension may exert via both glomerular and tubular function. Morelloflavone relaxes vascular smooth muscle of the thoracic aorta isolated from normotensive rats (Lamai et al. 2013), thus, it is likely that it may relax the afferent arterioles resulting in an increased GFR. It is also feasible that morelloflavone might exert its diuretic effect by inhibition of epithelial electrolyte transport in either proximal or distal part of the nephron. Other plant flavonoids that elicit renal vasodilation and increase urinary sodium and water excretion including the flavonoids extracted from *Clerodendron trichotomum* and *Spergularia purpurea* (Jouad et al. 2001; Lu et al. 1994).

The similar diuretic action of morelloflavone was observed by an increase in and CO_{sm} in 2K1C (Figure 3(e) and 3(g)) but not by a significant GFR alteration (Figure 3(d)). These indicated the inhibition of renal tubular electrolyte reabsorption by morelloflavone rather than its increased filtered load action like those in SO rats. In addition, in SO rats, morelloflavone did not alter tubular free water reabsorption which contradicted to 2K1C rats (Figure 3(h)) suggesting another different pathway of its diuretic action in hypertensive model. Distal tubular water reabsorption occurs mainly in response to antidiuretic hormone (ADH) action, so it is possible that morelloflavone can improve water reabsorption by increasing the expression of renal tubular water channel, aquaporin in this model.

CONCLUSION

This work is the first preliminary report of the diuretic and hypotensive effect of morelloflavone, a plant biflavonoid, in hypertensive rats. Its hypotensive effect and the restoration of an impaired BRS may occur via vasorelaxant property of morelloflavone that enhances NO bioavailability. The diuretic action of morelloflavone is likely to involve both increased glomerular filtration and decreased tubular reabsorption of electrolytes and water.

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