Original Article

Flax lignan concentrate attenuate hypertension and abnormal left ventricular contractility via modulation of endogenous biomarkers in two-kidney-one-clip (2K1C) hypertensive rats

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A B S T R A C T

The present investigation was designed to study the effect of flax lignan concentrate obtained from *Linum usitatissimum* L., Linaceae, in two-kidney, one clip (2K1C) hypertension model in Wistar rats. 2K1C Gold-blatt model rats were divided randomly into six groups: sham, 2K1C control, captopril (30 mg/kg), flax lignan concentrate (200, 400 and 800 mg/kg). Flax lignan concentrate and captopril were administered daily for eight consecutive weeks. Sham-operated, and 2K1C control rats received the vehicle. Treatment with flax lignan concentrate (400 and 800 mg/kg) significantly and dose-dependently restored the hemodynamic parameters systolic blood pressure, diastolic blood pressure, mean arterial blood pressure and left ventricular functions. The flax lignan concentrate significantly restored the elevated hepatic, renal and cardiac marker enzymes in the serum. It also restored the organs weights (kidney and heart), serum electrolyte level and histological abnormalities. Furthermore, flax lignan concentrate significantly elevated the level of biochemical markers that is enzymatic antioxidants superoxide dismutase, glutathione and decreased malondialdehyde in the heart and kidney tissues. Meanwhile, we found that plasma nitric oxide and plasma nitric oxide synthase contents were significantly increased in the flax lignan concentrate-treated group, and plasma endothelin-1 and renal angiotensin-II levels were significantly lower than 2K1C hypertensive group. In conclusion, the antihypertensive and antioxidant effect of flax lignan concentrate were dose-dependent and at the highest dose (*i.e.* 800 mg/kg) similar to those of captopril (30 mg/kg). It is suggested that flax lignan concentrate reduced blood pressure by reduction of renal angiotensin-II level, inhibition of plasma endothelin-1 production, induction of the nitric oxide, nitric oxide synthase and *in vivo* antioxidant defense system.

Introduction

The one-third adult population of the world is affected by hypertension, and it can be considered as one of the most common chronic disease nowadays (Lee et al., 2009; Gosavi et al., 2011; Ghosh et al., 2012; Shivakumar et al., 2014). The world's adult population with hypertension is likely to increase from one billion in 2000 to 1.56 billion by 2025 (Kearney et al., 2005).

Renin–angiotensin–aldosterone system (RAAS) plays an important role in hypertension. Renal ischemia leads to secrete renin by kidney tissue which is responsible for catalyzing the hydrolysis of angiotensin-I (Ang-I) from the *N*-terminus of angiotensinogen. Ang-I is converted into Ang-II by the angiotensin-converting enzyme (ACE). Ang-II is the primary product of RAAS, which

leads to vasoconstriction and hypertension through binding to the Ang-II receptor and stimulating synthesis of aldosterone (Kamble et al., 2013; Badole et al., 2015). It is well known that Ang-II also stimulate the generation of superoxide anion radical ($O_2^{-•}$) (Griendling et al., 1994; Rajagopalan et al., 1996), which contribute to decreased nitric oxide (NO) bioavailability and impaired endothelium-dependent vasorelaxation (Gryglewski et al., 1986). Synthetic ACE inhibitors therapy is commonly used today to treat hypertension. However, this advanced antihypertensive therapy has serious side effects such as angioedema and dry cough (Coulter and Edwards, 1987). Therefore, the use of anantioxidant may be the possible therapy for the prevention and treatment of hypertension with the established antihypertensive drug (Gosavi et al., 2011, 2014; Visnagri et al., 2013).

Flaxseed or linseed (*Linum usitatissimum* L., Linaceae) is brown or yellow colored seed harvested from the blue flowers of flax crop. It has been used as food in India and around the world for a long time. Flaxseed mainly contains omega-3 fatty acid,

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α-linolenic acid, dietary fiber and secoisolariciresinol diglucoside (SDG) lignan (Bassett et al., 2009). Flaxseed contains ten to hundred times more lignan than most other edible plants seeds. It is reported that flaxseed also contains other lignans like matairesinol, lariciresinol, hinokinin, arctigenin, pinoresinol and demethoxy secoisolariciresinol in small quantity with several phenolic acid compounds (Prasad et al., 1998; Johnsson et al., 2000). Antidiabetic (Prasad et al., 2000), antihyperlipidemic (Raygude et al., 2012a), cardioprotective (Zanwar et al., 2011, 2013), renoprotective (Ghule et al., 2011, 2012, 2015), antiatherogenic (Prasad, 1997; Prasad et al., 1998), antioxidant, anticancer, antiviral, bactericidal and anti-inflammatory (Chen et al., 2002; Collins et al., 2003; Kinniry et al., 2006; Rajesha et al., 2006; Zanwar et al., 2010) potentials of flaxseed have been already reported.

Clinical studies involving patients with peripheral artery disease and high blood pressure (Rodriguez-Leyva et al., 2013; Khalesi et al., 2015) reported that consumption of flaxseeds in the diet for the duration of more than three months lowered blood pressure. The antihypertensive potential of flax lignan in chronic hypertensive condition has not been well explained in animals. The objective of the present work was to study the effect of flax lignan concentrate (FLC) in two- kidney, one-clip (2K1C) induced hypertension in Wistar rats.

Materials and methods

Experimental protocol

Male Wistar rats weighing (200–250 g) were purchased from National Toxicology Centre, Pune, India. They were maintained at $25 \pm 1\,°C$ temperature and 45–55% relative humidity under 12 h light/dark cycle. The animals had access to food pellets (manufactured by Pranav Agro Industries Ltd., Sangli, India) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), India. The IAEC approval number is CPCSEA/PCL/08/2014-15.

Drugs and chemicals

Captopril, sulfanilic acid, *N*-(1-naphthyl) ethylenediamine was purchased from Sigma–Aldrich Corporation, USA. Absolute alcohol (manufactured by Changshu Yangyuan Chemicals, China) was purchased from the respective vendor. Analytical grade hexane, hydrochloric acid, and sodium hydroxide were purchased from Qualigenes Fine Chemicals Pvt. Ltd., Mumbai, India.

Collection and authentication of plant seeds

Seeds of *Linum usitatissimum* L., Linaceae (flaxseeds) were obtained from Punjabrao Deshmukh Krishi Vidyapeeth, College of Agriculture, Nagpur, India. After the authentication of the seeds, a voucher specimen was deposited at our Institute, Poona College of Pharmacy, Pune, India. The flaxseeds were stored in a cold room before processing for oil extraction at our Real World Nutrition Lab, Bharati Vidyapeeth Deemed University, Pune, India.

Preparation of flax lignan concentrate (FLC)

Preparation of FLC was carried out as described previously (Zanwar et al., 2013). The flaxseed cake was defatted by hexane to remove residual oil. The defatted cake was then hydrolyzed with aqueous sodium hydroxide for 1 h at room temperature with intermittent shaking followed by extraction with 50% ethanol. The filtrate was acidified to pH 3 using 1 M hydrochloric acid. The filtrate was dried using rotavac apparatus at 50 °C. The dry powder of hydroalcoholic extract was labeled as FLC.

Preparation of drug solution and selection of FLC dose

Captopril and FLC were dissolved in distilled water. This study was carried out using three doses of FLC (*i.e.* 200, 400 and 800 mg/kg, *p.o.*) and one dose of captopril (*i.e.* 30 mg/kg, *p.o.*).

Experimental induction of hypertension

Wistar rats weighing 200–250 g were anesthetized with 50 mg/kg intraperitoneal administration of thiopental sodium. The fur on the back of each rat was shaved, and the skin was disinfected. A flank incision was made in the left lumbar area parallel to the long axis of the rat. The renal pedicel was exposed with the kidney retracted to the abdomen. Left renal artery was constricted to induce two-kidney, one-clip hypertension (2K1C), as previously described by Kharin and Krandycheva (2004). Briefly, a loop of the left renal artery was pulled into a segment of polyurethane tube [MRE 040-S20, Braintree Scientific; internal diameter (ID) = 0.50 mm, length 2 mm]. The muscle and skin layer (incision site) were sutured with a highly sterile suture needle. After one week of the recovery period, the animals were used for the further experiment. Rats in sham-operated group underwent the exposure of the left renal artery, but the artery was not constricted. The muscle and skin layer (incision site) were sutured with a sterile suture needle. After one week of the recovery period, the animals were used for the further experiment.

Experimental design

The rats were randomly divided into six groups, each containing six rats:

Group I: Sham-operated (vehicle distilled water *p.o.*)
Group II: 2K1C control (vehicle distilled water *p.o.*)
Group III: 2K1C + captopril (30 mg/kg *p.o.*)
Group IV: 2K1C + FLC (200 mg/kg *p.o.*)
Group V: 2K1C + FLC (400 mg/kg *p.o.*)
Group VI: 2K1C + FLC (800 mg/kg *p.o.*)

FLC and captopril were and administered to the rats orally using an oral feeding needle daily for eight consecutive weeks. The sham-operated and 2K1C control rats received vehicle distilled water. At the end of the study period, blood was collected by a retro-orbital puncture for the measurement of biochemical parameters.

Assessment of hemodynamic changes

Each rat was anesthetized with intraperitoneal injection of urethane (1.25 g/kg). The trachea was cannulated to assist respiration. The systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial blood pressure (MABP) were measured by invasive technique at the end of the eighth week. A polyethylene cannula (PE 50) filled with heparinized saline (100 IU/ml) was inserted into the right carotid artery. The cannula was connected to a transducer and the signal was amplified. The left ventricular hemodynamic changes were measuredusing a Millar mikro-tip transducer catheter (Model SRP-320; Millar Instrument, Inc. 320-7051, Houston, TX 77023-5417) inserted into the left ventricle via the right carotid artery and connected to a bioamplifier (Adil et al., 2015, 2016a; Visnagri et al., 2015). Maximum first derivative of ventricular pressure (dP/dt_{max}), minimum first derivative of ventricular pressure (dP/dt_{min}) and left ventricular end-diastolic

pressure (EDP) signals were obtained from primary signals (left ventricular systolic pressure and blood pressure) by means of Powerlab 8-channel data acquisition system (AD Instruments Pvt. Ltd., with Lab Chart 7.3 Prosoftware, Australia).

Sample collection and determination of biomarkers

Serum and plasma sample collection

At the end of the study period and 1 h after the test substance administration, the blood was collected by retro-orbital puncture under anesthesia. Serum samples were collected without added anticoagulant. Serum samples were collected after centrifugation for 10 min at $845 \times g$ and $4\,^{\circ}$C. The blood was collected into anticoagulant containing tubes and immediately centrifuged (10 min at $845 \times g$ and $4\,^{\circ}$C temperatures) for plasma sample collection. The serum and plasma samples were stored at $-80\,^{\circ}$C until being analyzed.

Heart and renal tissue samples

At the end of the experimental period, all the rats were humanely euthanized. The heart and kidneys were removed for further experiments. The portions of the heart and clipped renal tissues were individually homogenized in 10% cold Tris–hydrochloride buffer (10 mmol/l, pH 7.4) using tissue homogenizer (Remi, India) and centrifuged at $5283 \times g$ for 15 min at $0\,^{\circ}$C. The clear supernatant collected after the centrifugation was used for biochemical and molecular estimations.

Measurement of biological serum markers

The serum electrolytes such as Na^+, K^+ and Cl^- were estimated using commercially available measurement kits (Coral Clinical System, Goa, India). Creatine kinase (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein, blood urea nitrogen (BUN), uric acid and creatinine were also measured by using commercially available measurement kits (Accurex Pvt. Ltd., Mumbai, India).

Estimation of endogenous antioxidant enzyme

The superoxide dismutase (SOD) concentration was determined by the method previously described elsewhere (Kamble et al., 2013; Adil et al., 2014; Aswar et al., 2015; Honmore et al., 2015). The SOD activity was expressed as U/mg of protein. The glutathione (GSH) assay was performed according to the method previously describe elsewhere (Moron et al., 1979; Kandhare et al., 2011a, 2015a; Kumar et al., 2014; Ketkar et al., 2015; Goswami et al., 2016; Adil et al., 2016b). The amount of reduced glutathione was expressed as μg/mg of protein. Malondialdehyde (MDA) level in the kidney and heart tissues were measured by the method previously described elsewhere (Slater and Sawyer, 1971; Patil et al., 2011, 2015; Raygude et al., 2012a,b; Saraswathi et al., 2014; Kandhare et al., 2016a), and the values were expressed in nanomoles of MDA/mg of protein.

Determination of nitric oxide (NO), nitric oxide synthase (NOS), endothelin-1 (ET-1), Ang-II level

NO is highly unstable free radical, which is converted into stable metabolites nitrate and nitrite in the equimolar ratio (Schlaich et al., 2007; Visnagri et al., 2014; Kandhare et al., 2016a, 2015b; Sarkar et al., 2015). The plasma NO level was determined as nitrite by the acidic Griess reaction. The assay was performed by a rapid, simple spectrophotometric method described elsewhere (Miranda et al., 2001; Gosavi et al., 2012a,b; Kandhare et al., 2013a, 2014a). The principle of this assay is a reduction of nitrate by vanadium. The nitrite reacts with sulfonamide and N-(1-naphthyl)ethylenediamine to produce a pink azo-product with maximum absorbance at

543 nm. The concentrations were calculated using a standard curve of sodium nitrate and the results were expressed in μmol/l. ET-1, NOS [Genxbio Health Sciences Ltd., India] in the plasm and Ang-II level [RayBiotech, Inc., USA] in the renal tissue homogenate were measured using Elisa kits as per the instructions are given by the manufacturer.

Histopathological examination

The excised heart and kidney samples were cleaned and immediately fixed in neutral buffered 10% formalin solution. The specimens were routinely processed and embedded in paraffin. The specimens were cut in sections of $5\,\mu$m thickness by microtome and stained with Masson's trichrome for microscopic examination. The sections were observed under the microscope and photomicrographs of the tissue section were taken using a microscope camera (Nikon Cool pix). The parameters of histopathological assessment of the kidney sections were mainly perivascular edema, fibrosis, glomerular necrosis and collagen deposition. The parameters of histopathological assessment of the heart sections were myocardial degeneration, collagen deposition, and fibrosis.

Statistical analysis

The data were expressed as mean $\pm$ standard error of mean (SEM) and statistical analysis was carried out by one-way ANOVA followed by post hoc Dunnett's test using Graph Pad Prism 5.0 software (Graph Pad Software, San Diego, CA, USA). Differences with a value of $p < 0.05$ were considered statistically significant.

Results

Effect of FLC on hemodynamic parameters and left ventricular contractile function of heart

Fig. 1 presents the effect of three different concentrations (200, 400 and 800 mg/kg) on hemodynamic parameters and left ventricular contractile function in all the I–VI groups after 8 weeks. Compared to sham operated group, the rats in 2K1C control group showed significant ($p < 0.001$ each) increase in SBP, DBP, MABP, EDP, dP/dt_{max} and dP/dt_{min} after 8 weeks. Captopril (30 mg/kg) and FLC (800 mg/kg) treatment showed a significant ($p < 0.001$ each) decrease in the SBP, DBP, MABP, EDP, dP/dt_{max} and dP/dt_{min}. FLC (400 mg/kg) treatment also showed significant ($p < 0.01$ each) decrease in SBP, DBP, MABP, EDP, dP/dt_{max} and dP/dt_{min} compared to 2K1C-control group. Treatment with FLC (200 mg/kg) showed significant ($p < 0.05$) decrease in SBP, and dP/dt_{min}; but did not show any significant decrease in DBP, MABP, EDP and dP/dt_{max} values (Fig. 1).

Effect of FLC on organs weight and electrolyte

The 2K1C-control hypertensive (group-II) rats showed significant ($p < 0.001$) increase in the weights of kidney and heart. The serum sodium ion (Na^+) and chloride ion (Cl^-) levels significantly increased while that of serum potassium ion (K^+) level decreased compared to sham. The treatments with captopril (30 mg/kg) and FLC (400, 800 mg/kg) reduced organs weight and restored those ions (sodium, chloride, and potassium) level to near normal (Table 1).

Effect of FLC on serum cardiac, hepatic and renal markers and serum total protein level

The activities of CK-MB, LDH, AST, ALT, ALP, total protein, BUN, uric acid and creatinine were significantly increased in 2K1C
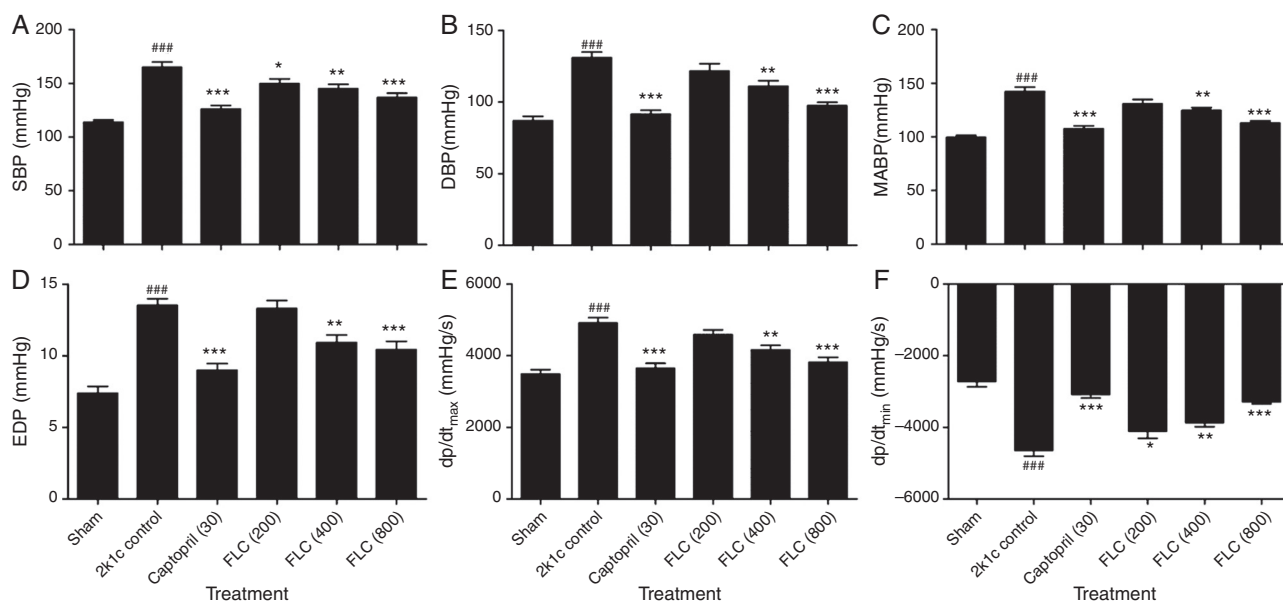
Fig. 1. Effect of administration of FLC and captopril on hemodynamic parameters (A) SBP, (B) DBP, (C) MABP, (D) EDP, (E) dP/dt_{max} and (F) dP/dt_{min} in modified 2K1C hypertensive Wistar rats ($n = 6$). Data are expressed as mean ± S.E.M. and statistical analysis was carried out by one-way ANOVA followed by *post hoc* Dunnett's test; ns = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with 2K1C control group-II, $^{#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$ compared to sham group-I.

Table 1
Effect of FLC (200, 400 and 800 mg/kg) and captopril (30 mg/kg) on organs (heart and kidney) weights and serum electrolytes in 2K1C hypertensive Wistar rats.

Parameter	Treatment				
	Sham	2K1C control	2K1C + captopril (30 mg/kg)	2K1C + FLC (200 mg/kg)	2K1C + FLC (400 mg/kg)	2K1C + FLC (800 mg/kg)
Organ weight (g)					
Heart weight	1.18 ± 0.03	1.54 ± 0.04[d]	1.20 ± 0.02[c]	1.44 ± 0.04[ns]	1.38 ± 0.05[b]	1.27 ± 0.02[c]
Kidney weight	1.41 ± 0.03	2.18 ± 0.05[d]	1.53 ± 0.04[c]	2.02 ± 0.05[ns]	1.94 ± 0.05[b]	1.62 ± 0.04[c]
Electrolyte (mEq/l)					
Sodium	144 ± 1.57	183 ± 6.29[d]	152 ± 3.39[c]	179 ± 5.45[ns]	161 ± 5.03[b]	155 ± 3.75[c]
Potassium	6.22 ± 0.22	3.67 ± 0.19[d]	5.73 ± 0.27[c]	3.81 ± 0.19[ns]	4.65 ± 0.20[b]	5.4 ± 0.15[c]
Chloride	104 ± 2.12	131 ± 4.21[d]	115 ± 1.66[c]	126 ± 2.5[ns]	120 ± 2.1[a]	116 ± 1.79[b]

Values are expressed as mean ± SEM for $n = 6$ rats. Data are expressed as mean ± S.E.M. and statistical analysis was carried out by one-way ANOVA followed by *post hoc* Dunnett's test; ns = non-significant.
[a] $p < 0.05$ as compared with 2K1C control (group-II).
[b] $p < 0.01$ as compared with 2K1C control (group-II).
[c] $p < 0.001$ as compared with 2K1C control (group-II).
[d] $p < 0.001$ compared to sham group (I).

Table 2
Effect of FLC (200, 400 and 800 mg/kg) and captopril (30 mg/kg) on serum cardiac, hepatic, renal markers and serum total protein in 2K1C hypertensive Wistar rats.

Parameter	Treatment				
	Sham	2K1C control	2K1C + captopril (30 mg/kg)	2K1C + FLC (200 mg/kg)	2K1C + FLC (400 mg/kg)	2K1C + FLC (800 mg/kg)
Aspartate aminotransferase (AST) (IU/l)	90.6 ± 3.21	162 ± 5.69[d]	108 ± 5.53[c]	144 ± 4.09[ns]	136 ± 4.96[b]	121 ± 3.97[c]
Alanine aminotransferase (ALT) (IU/l)	37.5 ± 2.13	75.3 ± 1.82[d]	50.8 ± 2.75[c]	68.6 ± 2.37[ns]	62.5 ± 1.38[b]	59.9 ± 3.05[c]
Alkaline phosphatase (ALP) (IU/l)	73.1 ± 4.54	186 ± 6.98[d]	129 ± 13.6[c]	166 ± 8.22[ns]	140 ± 9.48[b]	140 ± 10[b]
Total protein (mg/dl)	5.31 ± 0.22	8.50 ± 0.33[d]	6.01 ± 0.27[c]	7.54 ± 0.24[ns]	7.00 ± 0.26[b]	5.98 ± 0.29[c]
Blood urea nitrogen (BUN) (mg/dl)	17.80 ± 0.70	36.10 ± 1.81[d]	23.80 ± 1.38[c]	31.10 ± 1.05[a]	29.40 ± 1.27[b]	27.20 ± 1.05[c]
Uric acid (mg/dl)	1.93 ± 0.08	3.90 ± 0.15[d]	2.22 ± 0.07[c]	3.52 ± 0.10[a]	3.38 ± 0.10[b]	2.98 ± 0.09[c]
Creatinine (mg/dl)	0.77 ± 0.07	1.83 ± 0.04[d]	0.90 ± 0.05[c]	1.49 ± 0.12[ns]	1.36 ± 0.14[b]	1.12 ± 0.09[c]
Creatinine kinase (CK-MB) (IU/l)	345 ± 13.9	563 ± 16.6[d]	464 ± 14.1[c]	531 ± 11.4[ns]	500 ± 10.6[b]	484 ± 11.5[c]
Lactatedehydrogenase (LDH) (IU/l)	655 ± 10.6	770 ± 10.7[d]	672 ± 8.1[c]	732 ± 12.6[a]	712 ± 9.72[b]	680 ± 5.71[c]

Values are expressed as mean ± SEM for $n = 6$ rats. Data are expressed as mean ± S.E.M. and statistical analysis was carried out by one-way ANOVA followed by *post hoc* Dunnett's test; ns = non-significant.
[a] $p < 0.05$ as compared with 2K1C control (group-II).
[b] $p < 0.01$ as compared with 2K1C control (group-II).
[c] $p < 0.001$ as compared with 2K1C control (group-II).
[d] $p < 0.001$ compared to sham group (I).

Table 3
Effect of FLC (200, 400 and 800 mg/kg) and captopril (30 mg/kg) on endogenous antioxidant enzymes in 2K1C hypertensive Wistar rats.

Parameter	Treatment					
	Sham	2K1C control	2K1C + captopril (30 mg/kg)	2K1C + FLC (200 mg/kg)	2K1C + FLC (400 mg/kg)	2K1C + FLC (800 mg/kg)
SOD (Unit/mg protein)						
Kidney	12.47 ± 0.27	5.58 ± 0.21[d]	9.16 ± 0.34[c]	6.61 ± 0.34[ns]	7.21 ± 0.44[b]	8.62 ± 0.32[c]
Heart	$4.6d7 \pm 0.39$	3.04 ± 0.08[d]	4.25 ± 0.11[c]	3.32 ± 0.17[ns]	4.13 ± 0.11[b]	4.22 ± 0.08[c]
MDA (nmol of MDA/mg protein)						
Kidney	3.42 ± 0.23	7.93 ± 0.31[d]	4.33 ± 0.28[c]	6.95 ± 0.17[a]	6.44 ± 0.20[b]	5.45 ± 0.29[c]
Heart	4.35 ± 0.24	8.06 ± 0.23[d]	5.86 ± 0.09[c]	7.80 ± 0.16[ns]	6.91 ± 0.13[b]	6.05 ± 0.25[c]
GSH (μg/mg protein)						
Kidney	8.11 ± 0.26	4.58 ± 0.26[d]	6.41 ± 0.21[c]	4.94 ± 0.28[ns]	5.66 ± 0.18[b]	5.99 ± 0.15[c]
Heart	7.76 ± 0.36	3.91 ± 0.24[d]	6.74 ± 0.20[c]	3.86 ± 0.23[ns]	5.14 ± 0.15[b]	5.34 ± 0.18[c]

Values are expressed as mean $\pm$ SEM for $n = 6$ rats. Data are expressed as mean $\pm$ S.E.M. and statistical analysis was carried out by one-way ANOVA followed by *post hoc* Dunnett's test; ns = non-significant.

[a] $p < 0.05$ as compared with 2K1C control (group-II).
[b] $p < 0.01$ as compared with 2K1C control (group-II).
[c] $p < 0.001$ as compared with 2K1C control (group-II).
[d] $p < 0.001$ compared to sham group (I).

hypertensive rats (2K1C-control group-II). The treatment with captopril (30 mg/kg) and FLC (200, 400, 800 mg/kg) showed a reduction in the activities of these cardiac, hepatic and renal markers toward near normal. Captopril (30 mg/kg) showed the highest activity than the test drug FLC (200, 400, 800 mg/kg). FLC (800 mg/kg) showed significant ($p < 0.001$) reduction in serum level of all markers, except in ALP ($p < 0.01$). Treatment with FLC (400 mg/kg) also showed significant ($p < 0.01$) reduction in serum level of all markers. However, FLC in low dose (200 mg/kg) did not show any significant inhibition in the level of serum CK-MB, LDH, AST, ALT, ALP, total protein, and creatinine; but showed a significant reduction in serum level of BUN and uric acid compared to the 2K1C control animals (Table 2).

Endogenous antioxidant enzymes

The SOD and GSH activity in the tissues (kidney and heart) of 2K1C hypertensive rats were decreased significantly ($p < 0.001$) after 8 weeks. Captopril (30 mg/kg), as well as FLC (400, 800 mg/kg), restored the SOD and GSH activity in the tissues (kidney and heart) after eight weeks. On the other hand, FLC (200 mg/kg) did not show any significant restoration of the SOD and GSH activity in the tissues. MDA level in the tissues (kidney and heart) of 2K1C hypertensive rats increased significantly after 8 weeks. Captopril (30 mg/kg) and FLC (800 mg/kg) treated groups had significantly decrease the level of MDA than in the FLC (400 mg/kg) treated group. However, FLC (200 mg/kg) treated group did not show any significant restoration of MDA level (Table 3).

Effect of FLC on NO, NOS, Ang-II and ET-1 level

The 2K1C-control rats showed a significant decrease in plasma NO and NOS level as compared to the sham operated group rats. The rats treated with captopril (30 mg/kg) and FLC (400 and 800 mg/kg) significantly elevated plasma NO and plasma NOS levels. However, 2K1C hypertensive rats treated with FLC (200 mg/kg) did not show any significant effect (Fig. 2A and B).

The increased blood pressure in the 2K1C hypertensive rats was also linked with significantly increased kidney Ang-II levels compared with sham-operated group. Captopril (30 mg/kg) and FLC (400 and 800 mg/kg) treatment in 2K1C hypertensive rats showed decreases in Ang-II levels of the kidney (Fig. 2C). Plasma ET-1 level was elevated significantly in the 2K1C-control group

compared with sham group. Captopril (30 mg/kg) and FLC (400 and 800 mg/kg) significantly decreased the plasma ET-1 level dose-dependently toward near normal level (Fig. 2D).

Effect of FLC on histopathology of kidney

The histopathological examination of kidney tissues in the sham-operated rats showed normal glomerulus cell and tubuli with the absence of perivascular edema, fibrosis, and collagen deposition. On the other hand, the hypertensive 2K1C group rats showed a significant increase in perivascular edema, fibrosis, and collagen deposition. Captopril (30 mg/kg) and FLC (400 and 800 mg/kg) showed a decrease in perivascular edema, fibrosis, collagen deposition, and necrosis. However, FLC (200 mg/kg) did not show significant protection from hypertensive damage (Fig. 3).

Effect of FLC on histopathology of heart

The 2K1C hypertensive group rats showed severe myocardial degeneration, hypertrophy, and fibrosis. Captopril (30 mg/kg) treated group showed minimal myocardial degeneration and collagen deposition and fibrosis. FLC (400 and 800 mg/kg) treatment also showed a decrease in myocardial degeneration and collagen deposition and fibrosis. However, FLC (200 mg/kg) did not show any significant protection (Fig. 4).

Discussion

The 2K1C is a classical method to induce hypertension in rats similar to human, which is primarily based on RAAS (Thurston et al., 1980; Ponchon and Elghozi, 1996; Kandhare et al., 2011b). The main mechanism behind the 2K1C hypertension is RAAS rather than a disturbance in kidney function (Nogueira et al., 2012). Unilateral renal artery occlusion decreases perfusion pressure inside the kidney and stimulates renin synthesis, which then produce Ang-II and increases the peripheral resistance and blood pressure (Pickering, 1989).

Our study demonstrated that higher doses of FLC (400 and 800 mg/kg) significantly decreased systolic, diastolic and mean arterial blood pressures in the 2K1C hypertensive Wistar rats. Secoisolariciresinol diglucoside (SDG), a main constituent of FLC, is reported to have a similar type of results in normotensive and Ang-I induced acute hypertensive animals. It is postulated that
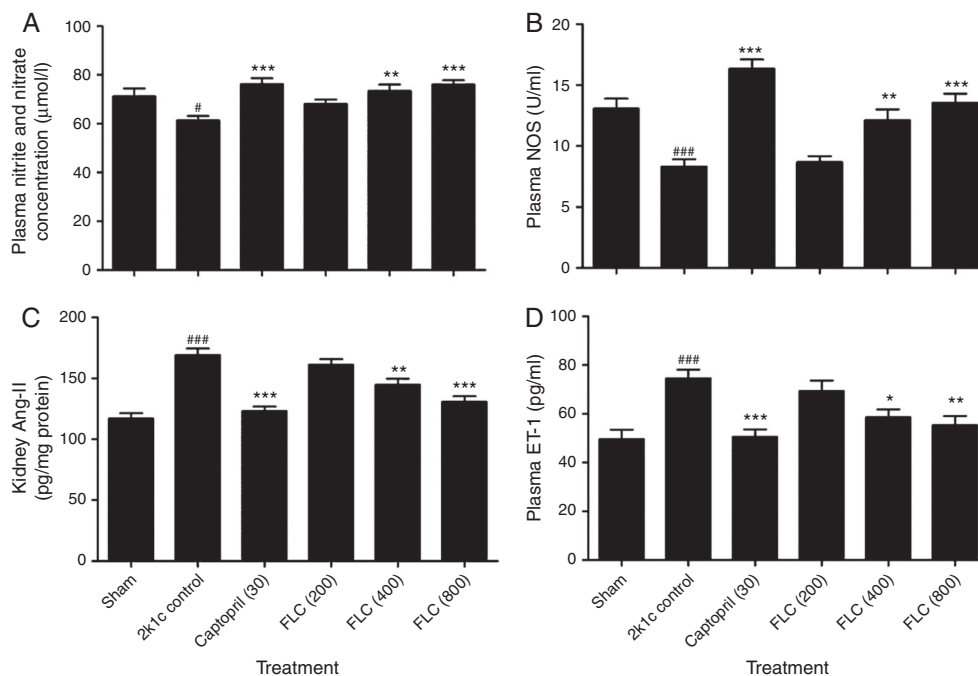
Fig. 2. Effect of administration of FLC and captopril on plasma NO levels (A), plasma NOS activity (B), Ang-II of clipped kidney (C) and plasma ET-1 (D) in 2K1C hypertensive Wistar rats ($n = 6$). Data are expressed as mean ± S.E.M. and statistical analysis was carried out by one-way ANOVA followed by *post hoc* Dunnett's test; ns = non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared with 2K1C control group-II, $^\#p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to sham group-I.
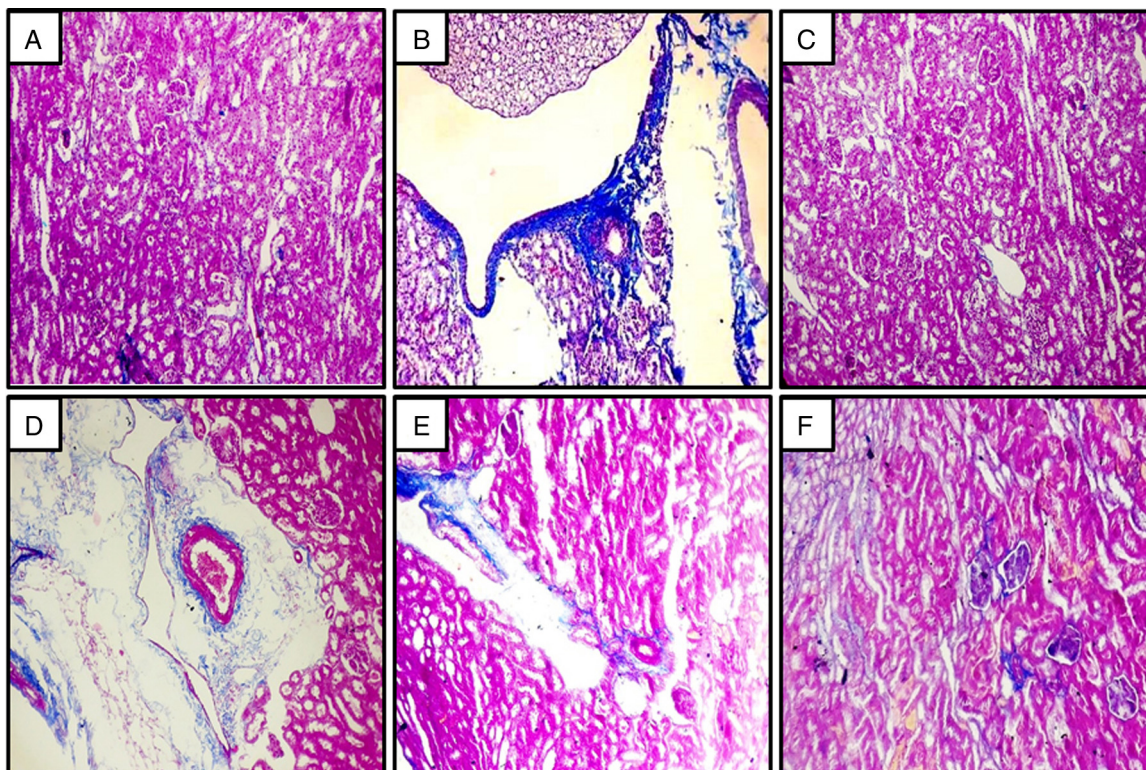

Fig. 3. Effect of administration of FLC and captopril on kidney histology in 2K1C hypertensive Wistar rats. Photomicrograph of sections of lungs of (A) Sham-operated rats (group-I) showed normal glomerulus cell and tubular with the absence of perivascular edema and collagen deposition. (B) 2K1C control rats (group-II) showed a significant increase in collagen deposition and perivascular edema, necrosis. (C) 2K1C + captopril (30 mg/kg) (group-III) showed a decrease in collagen deposition, atrophy, and necrosis. (D) 2K1C + FLC (200 mg/kg) (group-IV) showed no significant protection against hypertension (E) 2K1C + FLC (400 mg/kg) (Group V) showed decrease in collagen deposition and perivascular edema, glomerulus necrosis (F) 2K1C + FLC (800 mg/kg) (Group VI) showed significantly reduced perivascular edema, fibrosis, glomerular necrosis and collagen deposition (Masson's trichrome 20×).
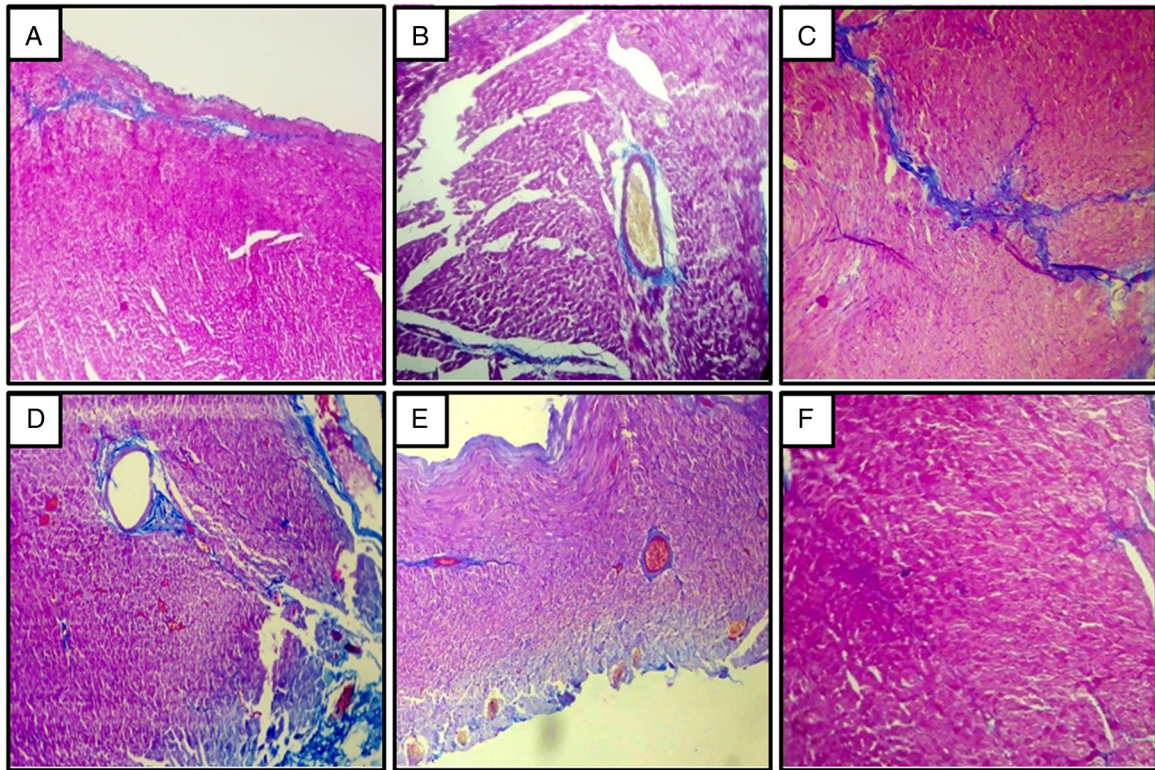
Fig. 4. Effect of administration of FLC and captopril on heart histology in 2K1C hypertensive Wistar rats. Photomicrograph of sections of lungs of (A) sham-operated rats (group-I) showed normal cardiac muscle fibers with the absence of myocardial fibrosis. (B) 2K1C control (group-II) showed severe myocardial degeneration, hypertrophy, and fibrosis. (C) 2K1C+ Captopril (30 mg/kg) (group-III) showed minimal myocardial degeneration and collagen deposition and myocardial fibrosis. (D) 2K1C + FLC (200 mg/kg) (group-IV) showed no significant decrease in collagen deposition and myocardial fibrosis. (E) 2K1C + FLC (400 mg/kg) (Group V) showed a decrease in myocardial degeneration and collagen deposition and fibrosis. (F) 2K1C + FLC (800 mg/kg) (Group VI) showed a significant reduction in myocardial degeneration, collagen deposition, and fibrosis (Masson's trichrome 20×).

the activity of SDG may be due to the stimulation of guanylate cyclase-nitric oxide pathway and by inhibition of angiotensin converting enzyme (ACE) (Prasad, 2004; Kamble et al., 2013). Earlier, we have determined SDG content in FLC by using high-performance liquid chromatography (HPLC) analysis and reported blood pressure lowering effect of FLC in DOCA-salt-induced renal hypertension model in rats (Sawant and Bodhankar, 2016). In the present study, the effect of FLC on the renin–angiotensin system dependent 2K1C hypertensive rat, similar to those of captopril, lead us to consider that SDG a main constituent from FLC may possess ACE-inhibitor-like properties. However, the possibility of a potentiating effect of SDG by other flavonoids and minor constituents present in FLC cannot be ruled out.

The hemodynamic data showed that left ventricle end diastolic pressure, max dP/dt and min dP/dt increased in 2K1C hypertensive rats, which are a clear sign of increased preload and afterload in the heart. The altered left ventricular parameters in 2K1C hypertensive rats also showed decrease contractility, diastolic compliance and dysfunction in the heart (Wang et al., 2007; Junhong et al., 2008). FLC and captopril are restored EDP, max dP/dt and min dP/dt significantly indicated that FLC and captopril decreased the burden on the heart, increased the conformity of myocardium and improved cardiac function. These findings thus support that FLC containing SDG as main constituent has antihypertensive potential in 2K1C hypertensive rats.

Chronic hypertension leads to continuous accumulation of interstitial collagen fibers and an increase in heart weight (Rossi and Peres, 1992). It is proved that Ang-II of RAAS is also involved in the tissue hypertrophy or hyperplasia. Therefore, RAAS plays an important role in the weight increase of heart and kidney in the 2K1C hypertensive model (Kobayashi et al., 1999). The current

results showed the captopril and FLC significantly prevent the increase in kidney and heart weight associated with hypertrophy, which may be due to antihypertensive effects of captopril and FLC.

It is well known that intracellular sodium ion concentration increases and potassium ion concentration decreases significantly in hypertension (Adrogue and Madias, 2007). Our results are thus, in accordance with the previous study and suggested that restoration of serum sodium and potassium ion may be due to antihypertensive effects of FLC.

The liver plays an important role in metabolism, toxicity and elimination of endogenous and exogenous elements. Liver damage leads to increased activity of AST, ALT, ALP and total protein in the plasma (Navarro et al., 1993; Bhattacharjee et al., 2009; Visnagri et al., 2012; Kandhare et al., 2013b,c, 2015d; Sarkate et al., 2015; Devkar et al., 2016). The reason behind the elevation of AST, ALT, ALP and total protein in the 2K1C hypertension may be oxidative stress that caused leakage of these enzymes from liver tissues due to membrane damage. After the administration of FLC and captopril, there was a significant decrease in the serum activities of AST, ALT, ALP and total protein that clearly signifies that captopril and FLC protected the functional capacity of liver and prevented oxidative damage due to hypertension.

In hypertension, volume and pressure loads on the kidneys lead to the dysfunction and damage to the renal tissues (Möhring et al., 1975; Kandhare et al., 2015d). Blood urea nitrogen (BUN), serum creatinine, and uric acid are considered as markers of the renal function. They are produced due to disturbance of protein and nucleic acid metabolism in the hypertensive stress. Several animal studies of 2K1C hypertension have reported that hypertension elevates the levels of blood urea nitrogen, serum creatinine and uric acid (Kang et al., 2002; Amat et al., 2014). The oxidative stress caused

due to high blood pressure may be the reason for the elevated levels of BUN, creatinine, and uric acid in the serum (Kandhare et al., 2012a,b,c,d, 2015c). The current study showed a reduction in the elevated serum level of renal markers by FLC and explain its protective effect in 2K1C hypertensive rats. Hypertension induces myocardial damage, and the serum levels of CK-MB and LDH are considered as standard markers for the identification of cardiac damage (Mair et al., 1994). The decreased serum level of these enzymes in the FLC-treated groups offered protection to heart in the 2K1C hypertension.

The present study showed that the activity of SOD and GSH in the heart and kidney tissues of 2K1C control group was lower than that in the sham-operated group. On the other hand, MDA activity in the 2K1C control group was significantly higher than in the sham-operated group. Our results thus suggested that hypertension led to increased oxidative stress, which is in agreement with the previous study (Cao et al., 2013). The current study showed that FLC and captopril administration scavenging the oxygen free radical in the blood leading to their anti-oxidant effects.

Blood flow-induced shear stress on endothelial cells plays key role in the production of NO by the endothelial NOS (Pohl et al., 1986; Kandhare et al., 2012d, 2014b, 2016b). NO is vasorelaxant substance and physiological antagonist of the Ang-II of RAAS at vascular levels. Ang-II reduces NO bioavailability by promoting superoxide anion, which is responsible for vasoconstriction and increase in blood pressure (De Nicola et al., 1992; Cosentino et al., 1994; Zhou et al., 2014).

The antagonistic effects of NO and Ang-II come out also in interactions with other vasoactive substance ET-1, which is the potent vasoconstrictor released from the endothelium (Rubanyi, 1994; Zhou et al., 2014). ET-1 is overexpressed in the vasculature and blood in various models of hypertension, including the 2K1C model, which increases systemic blood pressure (Iglarz and Schiffrin, 2003; Cao et al., 2013). In the present study, these biomarkers were altered in the 2K1C control hypertensive rats, where simultaneously the blood pressure increased with Ang-II and ET-1, as a synthesis of NO and NOS decreased. All these changes were opposed significantly by the ACE inhibitor drug captopril and FLC. Dose-dependent effects of FLC on the renin–angiotensin system markers were similar to those of captopril and suggest ACE inhibitor like property of FLC.

Histological study of the rat heart and kidneys revealed that the FLC treatment in the 2K1C hypertensive rats reduced cardiac and renal damage correlate with the various hemodynamic, biochemical observations. These results support the antihypertensive activity of FLC in the 2K1C hypertensive rats.

Conclusion

Antihypertensive and antioxidant effects of FLC in 2K1C hypertension were dose-dependent and at the highest dose (800 mg/kg) similar to those of captopril, which is mainly characterized by the reduction in blood pressure, restoration of altered left ventricular functions and endogenous biomarkers. It is concluded that FLC may reduce blood pressure by reduction of kidney Ang-II level, inhibition of ET-1 production and induction of the NO, NOS and *in vivo* antioxidant defense system.

Ethical statement

The animal experiments in the present work were carried out strictly following the guidelines given by CPCSEA, India. The protocol was approved by the Institutional Animal Ethical Committee (IAEC) constituted as per guidelines of CPCSEA.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

SS contributed in collecting plant sample and identification, a confection of the herbarium, running the laboratory work, analysis of the data and drafted the paper. SLB supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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