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RESEARCH ARTICLE

Carthamus Tinctorius L. extract attenuates cardiac remodeling in L-NAME-induced hypertensive rats by inhibiting the NADPH oxidase-mediated TGF-β1 and MMP-9 pathway

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ABSTRACT

Carthamus tinctorius L. (CT) has been widely used in Asian countries as a beverage and a folk medicine. The current study investigates the effect of CT extract on cardiac remodeling and possible mechanisms involved in N_w-nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced hypertensive rats. Male Sprague-Dawley rats were administrated with L-NAME (40 mg/kg/day) for five weeks to induce hypertension. Hypertensive rats were treated with CT extract (300 mg/kg/day) or captopril (5 mg/kg/day) or vehicle for a further two weeks. Treatment of hypertensive rats with CT extract or captopril significantly decreased systolic blood pressure, left ventricular (LV) hypertrophy and fibrosis, small intramyocardial coronary artery remodeling, and cardiac weight index. CT extract or captopril increased plasma nitric oxide metabolite (NOx) levels and reduced plasma transforming growth factor β1 (TGF-β1) level, together with downregulation of cardiac TGF-β1 and matrix metalloproteinases-9 (MMP-9) expression. In addition, decreased plasma malondialdehyde (MDA) levels, consistent with downregulation of NADPH oxidase subunit $gp91^{phox}$ expression in heart tissue, was also observed after CT extract or captopril treatment. These findings suggest that CT extract alleviates cardiac remodeling in L-NAME-induced hypertensive rats, which is possibly related to inhibition of the NADPH oxidase-mediated TGF-β1-MMP-9 pathway.

1. Introduction

Hypertension, or high blood pressure, is an important risk factor for various cardiovascular diseases and its prevalence is rapidly increasing worldwide (Kearney et al., 2005). Chronic hypertension can cause both cardiac hypertrophy and fibrosis, resulting in left ventricular (LV) structural remodeling and a progressive decline in LV performance (Kurrelmeyer et al., 1998). Chronic inhibition of nitric oxide (NO) synthesis by the administration of N_w-nitro-L-arginine methyl ester hydrochloride (L-NAME) has been reported to produce high blood pressure, together with cardiovascular morphological alterations in rats. Recent studies have demonstrated that left ventricular (LV) hypertrophy and myocardial fibrosis develops in NO-deficient hypertensive rats induced by L-NAME (Bunbupha et al., 2015; Pechanova et al., 2004). In addition, intramyocardial coronary artery remodeling (increased wall-to-lumen ratio and perivascular fibrosis) is present in L-NAME-induced hypertensive rats (Sonoda et al., 2017).

Transforming growth factor β1 (TGF-β1) is a multifunctional cytokine that regulates various physiological and pathological processes, including tissue wound healing, cell proliferation, and extracellular matrix (ECM) synthesis (Faler et al., 2006; Hocevar and Howe, 2000). Matrix metalloproteinases-9 (MMP-9) play an important role in the degradation of ECM and represent potential biomarkers for cardiac remodeling after myocardial infarction (Halade et al., 2013). Currently, there is substantial evidence that overproduction of reactive oxygen species (ROS) stimulates TGF-β1 and MMP-9, resulting in tissue fibrosis. Oxidative stress occurs

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when NADPH oxidase (gp91^{phox} subunit) expression is upregulated, and results in endogenous ROS-enhanced TGF-β1 synthesis and cardiac fibrosis in hypertensive rats (Zhao et al., 2008). Furthermore, ROS are linked to fibrosis and matrix turnover involving the activation of MMP-9 (Siwik et al., 2001).

Recently, the prevention of cardiovascular diseases has been associated with the consumption of fresh fruits, vegetables or plants rich in natural antioxidant nutrients (Giugliano, 2000). *Carthamus tinctorius* L. (CT), commonly known as safflower, a member of the family Compositae or Asteraceae, is widely used as a traditional medicine. An increasing number of studies are reporting the chemical components and pharmacological activities of CT. Several antioxidant compounds, such as flavonoids, quinochalcones, alkaloids, and polyacetylenes have been isolated from CT (Zhou et al., 2014). The pharmacological activities of CT are reported to include antioxidant, anti-inflammatory, cardioprotective and antitumor activities (Fu et al., 2016; Han et al., 2009; Loo et al., 2004). A previous study has demonstrated that CT extract has antihypertensive activity in L-NAME-induced hypertensive rats (Maneesai et al., 2016). Additionally, CT extract alleviates hemodynamic alterations and vascular remodeling in a rat model of renovascular hypertension (Bunbupha et al., 2018). However, there is little information about the effects of CT extract on cardiac remodeling. The aim of this study was to investigate whether CT extract could suppress the NADPH oxidase-mediated TGF-β1-MMP-9 pathway and alleviate cardiac remodeling in hypertensive rats induced by L-NAME.

Captopril, an ACE inhibitor commonly used for hypertension treatment, was employed as a positive control in this study. Captopril has been reported to have antioxidant activity due to its thiol group (Pechanova, 2007), and suppresses production of reactive oxygen species (ROS) by NADPH oxidase (Bunbupha et al., 2018). Moreover, it effectively attenuated myocardial and perivascular fibrosis, and reduced the thickness of the intramyocardial coronary artery in L-NAME-induced hypertensive rats (Sonoda et al., 2017).

2. Materials and methods

2.1. Chemicals

Captopril, L-NAME, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), butylated hydroxytoluene (BHT), *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) and sulfanilamide were obtained from Sigma–Aldrich (St Louis, MO, USA). Nitrate reductase, nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate disodium and glucose-6-phosphate dehydrogenase were obtained from Roche Applied Sciences (Mannheim, Germany). Trichloroacetic acid (TCA) was obtained from Fluka Chemika (Buch, Switzerland). All chemicals used were of analytical grade quality.

2.2. Preparations of C. tinctorius L. (CT) extract

Dry CT was purchased from Vejpong Pharmacy, Co., Ltd. (Bangkok, Thailand). Dry CT (1000 g) was soaked in 95% ethanol (2000 mL) for 4 h. Then, it was filtered through nylon cloth and dried using a spray dry machine. The yield (calculated on the dried powder extract) was 11.25% of the dried CT.

2.3. Animals and experimental protocols

Male Sprague-Dawley rats, weighing 220–240 g, were procured from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. The animals were housed at 23 ± 2 °C with a 12 h dark–light cycle at Northeast Laboratory Animal Center, Khon Kaen University, Khon Kaen, Thailand. The experimental procedures were approved by the Animal Ethics Committee of Khon Kaen University (AEKKU-NELAC 5/2557). After a week of acclimatization, animals were randomly divided into four groups (eight rats in each group): Group I, control + vehicle (distilled water, 0.15 mL/100 g), Group II, L-NAME + vehicle, Group III, L-NAME + CT extract (300 mg/kg/day), and Group IV, L-NAME + captopril (5 mg/kg/day).

Over the 5-week study, control rats received distilled water, while hypertensive rats received L-NAME (40 mg/kg/day) in their drinking water in order to induce hypertension. CT extract, captopril or vehicle were intragastrically administered daily for the last 2 weeks of the study. The concentrations of CT extract and captopril used in this study were selected based on findings from a previous study (Maneesai et al., 2016).

2.4. Indirect measurement of blood pressure in conscious rats

Systolic blood pressure (SBP) of all animals was measured weekly using non-invasive tail-cuff method (IITC/Life Science Instrument model 229 and model 220 amplifiers; Woodland Hills, CA, USA). In brief, conscious rats were placed in a plastic restrainer. A cuff with a pneumatic pulse sensor was attached to the tail, and the cuff was automatically inflated and released. Rats were allowed to habituate to this procedure for 7 days before experiments were performed. The average of three measurements with 15 min intervals was used for data analysis.

2.5. Heart weights, tissue sampling and blood plasma isolation

At the end of the study, animals were anaesthetized by peritoneal injection of pentobarbital sodium (60 mg/kg). Blood samples were collected from the abdominal aorta and EDTA tubes were used to prevent coagulation. Blood was centrifuged at 3500 rpm and 4 °C for 15 min to separate plasma and then stored at −80 °C. After blood sampling, the animals were killed by overdose of the anaesthetic drug. Wet heart weight, right ventricular weight (RVW) and left ventricular weight (LVW) were measured, and LVW to body weight (BW) ratio (LVW/BW) and RVW to BW ratio (RVW/BW) were calculated. Samples of the LV were used for western blot and histological analysis.

2.6. Assay of plasma NO metabolite (NOx) concentration

Plasma NOx concentration was measured using an enzymatic conversion method (Verdon et al., 1995). Plasma samples were deproteinized by ultrafiltration using centrifugal concentrators (Pall Corp., Ann Arbor, MI, USA). The nitrate in the supernatant was reduced to nitrite by nitrate reductase, and then the mixture was reacted with Griess solution (4% sulfanilamide in 0.3% NED) for 15 min. The absorbance of samples was detected at a wavelength of 540 nm using a microplate reader (Tecan, Grodig, Austria).

2.7. Assay of plasma malondialdehyde (MDA) concentration

Plasma MDA concentration was used for estimation of lipid peroxidation by the TBA reactive substances method (Draper et al., 1993). This involved mixing 150 μL of plasma with 10% TCA, 5 mmol/L EDTA, 8% SDS, and 0.5 μg/mL BHT, and then incubating at room temperature for 10 min. Next, 0.6% TBA was added and the mixture was boiled in a water bath for 30 min. After cooling to room temperature, the mixture was centrifuged at 10,000 g for 5 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (Amersham Bioscience, Arlington, MA, USA).

Table 1
Effects of CT extract and captopril on cardiac mass indexes in all experimental groups.

Parameter	Control	L-NAME	L-NAME + CT	L-NAME + Cap
Body weight (g)	423.3 ± 6.6	419.9 ± 6.7	422.8 ± 5.7	411.8 ± 10.8
Heart weight/BW (mg/g)	3.25 ± 0.04	3.92 ± 0.08*	3.43 ± 0.03#	3.37 ± 0.05#
LVW/BW (mg/g)	2.26 ± 0.03	2.98 ± 0.12*	2.43 ± 0.08#	23.6 ± 0.06#
RVW/BW (mg/g)	0.66 ± 0.03	0.62 ± 0.04	0.63 ± 0.01	0.61 ± 0.03

Results are expressed as mean ± SEM.
* $p < 0.05$ vs. control group.
# $p < 0.05$ vs. L-NAME group ($n = 8$).

2.8. Assay of plasma transforming growth factor beta1 (TGF-β1) concentration

The concentration of plasma TGF-β1 was measured using an enzyme-immunoassay (ELISA) kit (ab119557, Abcam Plc, Cambridge, UK).

2.9. Histology and morphometry

The LV tissues were fixed for 24 h in 4% paraformaldehyde, processed routinely in paraffin, and cut to a thickness of 5 mm. LV hypertrophy and vessel medial thickness were assessed using hematoxylin and eosin of stained LV sections. Images were obtained under SMZ745T stereomicroscopes and a DS-2Mv light microscope (Nikon, Tokyo, Japan) respectively. The intramyocardial coronary arteries ranging in diameter from 50 to 150 μm were randomly selected and used for media/lumen ratios analysis. An average of 10 intramyocardial coronary arteries from four subserial sections was used as the value for each animal (Shi et al., 2007). The media/lumen ratios of the vessels (media/lumen ratios = media wall area/luminal area) was calculated, and expressed as the percentage of media/lumen ratios (Sawada et al., 2009). Myocardial interstitial and perivascular fibrosis were assessed using picrosirius red stained LV sections. Sections were captured with an Eclipse LV100 POL polarized light microscope (Nikon, Tokyo, Japan). LV fibrosis was expressed as a percentage of the positively stained area to medial area. Perivascular fibrosis was evaluated around coronary arterioles, which was expressed as the percentage of perivascular fibrotic area/(luminal area + medial area + perivascular fibrotic area) (Sawada et al., 2009). Morphometric evaluations were analyzed using ImageJ morphometric software (National Institutes of Health, Bethesda, MD, USA) and Image-pro plus software (Media Cybernetics, MD, USA).

2.10. Western blot analysis

Protein TGF-β1, MMP9 and gp91^{phox} expression were measured in heart tissue homogenates. Homogenates were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system. The proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane, and blocked with 5% skimmed milk in phosphate buffered saline with 0.1% Tween-20 (PBST) for 2 h at room temperature. Following this, they were incubated overnight at 4 °C with primary antibody against gp91^{phox} (BD Biosciences, San Jose, CA, USA), TGF-β1, MMP9 and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membranes were washed with PBST and then incubated for 2 h at room temperature with horseradish peroxidase conjugated secondary antibody. The blots were developed in Amersham ECL Prime solution (Amersham Biosciences, Piscataway, NJ, USA) and densitometric analysis was performed using an ImageQuant400 imager (GE Healthcare Life Science, Piscataway, NJ, USA). Band intensity was normalized to that of β-actin, and data were expressed as a percentage of the values determined in the control group from the same gel.

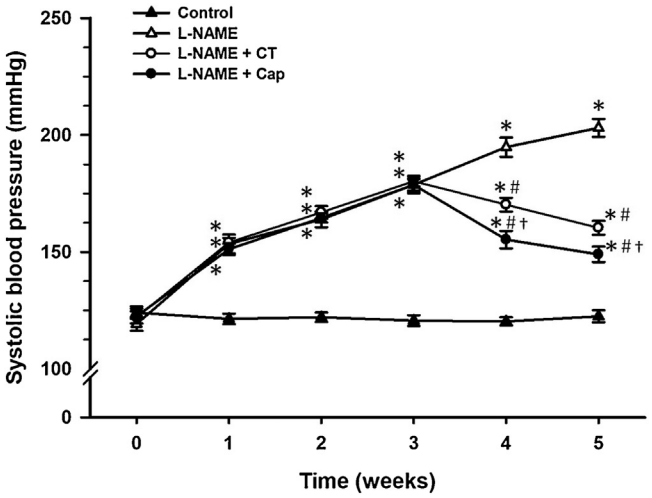

Fig. 1. Systolic blood pressure (indirect measurement) during treatments in all experimental groups. Results are expressed as mean ± SEM. *$p < 0.05$ vs. control group, #$p < 0.05$ vs. L-NAME group, †$p < 0.05$ vs. L-NAME + CT group ($n = 8$).

2.11. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Multiple comparisons between groups were made with one-way analysis of variance (ANOVA) with a Student–Newman–Keuls test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Effects of CT extract and captopril on SBP in conscious rats

At the beginning of the study, there were no significant differences in average baseline values of SBP between any of the groups of rats. In the control group, the SBP did not change throughout the 5 weeks of the experiment. Administration of L-NAME for 5 weeks caused a progressive increase in SBP compared with the control group (SBP at 5th week, 203.1 ± 3.9 mmHg versus 122.5 ± 2.5 mmHg; $p < 0.05$). After 2 weeks of treatment, both CT extract and captopril significantly reduced SBP in hypertensive rats compared to untreated hypertensive rats. The blood pressure lowering effects of captopril were significantly larger than those of CT extract (160.3 ± 3.0 mmHg and 148.9 ± 3.3 mmHg; $p < 0.05$; Fig. 1).

3.2. Effect of CT extract and captopril on cardiac mass indexes

At the conclusion of the 5 week experiment, there were no significant differences in animal BW between groups. Rats that received L-NAME showed increased heart weight/BW and LVW/BW ratio compared with the control group ($p < 0.05$; Table 1) without a significant change in the RVW/BW ratio. Hypertensive rats treated with CT extract or captopril showed a markedly reduced

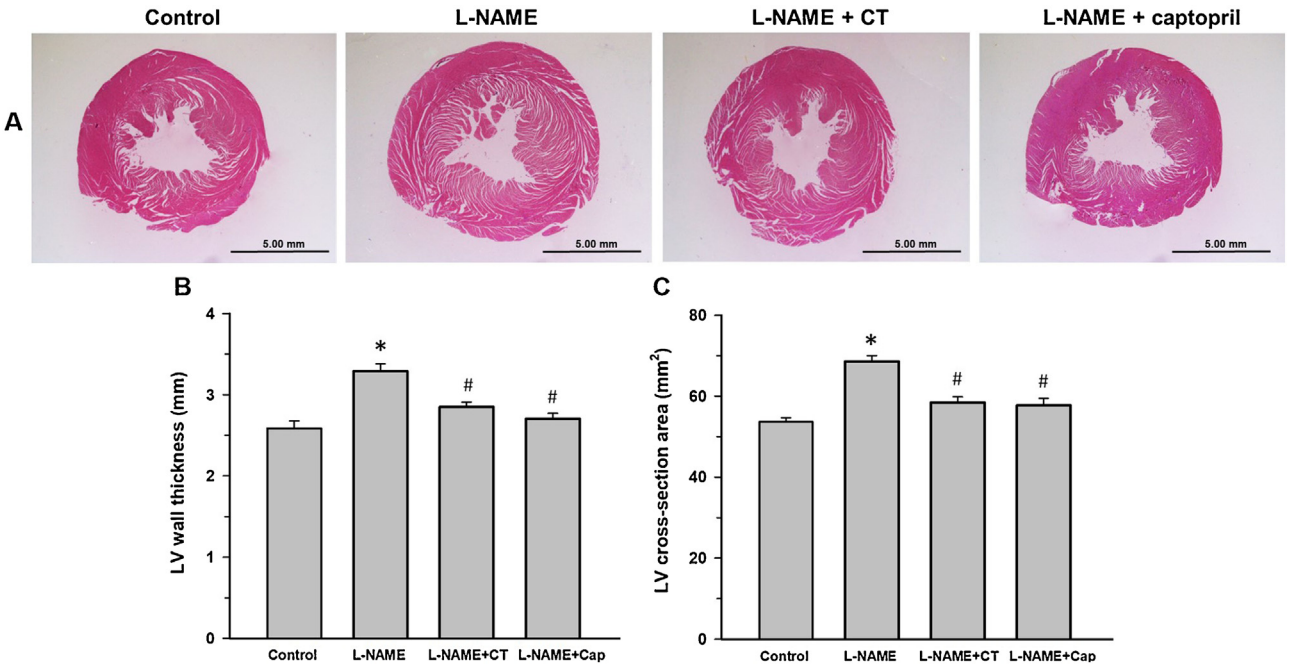

Fig. 2. Effects of CT extract and captopril on LV morphology in L-NAME-induced hypertensive rats. Representative images of LV sections stained with hematoxylin and eosin under stereomicroscopes (A), and values of LV wall thickness (B) and LV cross-sectional area (C). Results are expressed as mean ± SEM. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. L-NAME group ($n = 8$).

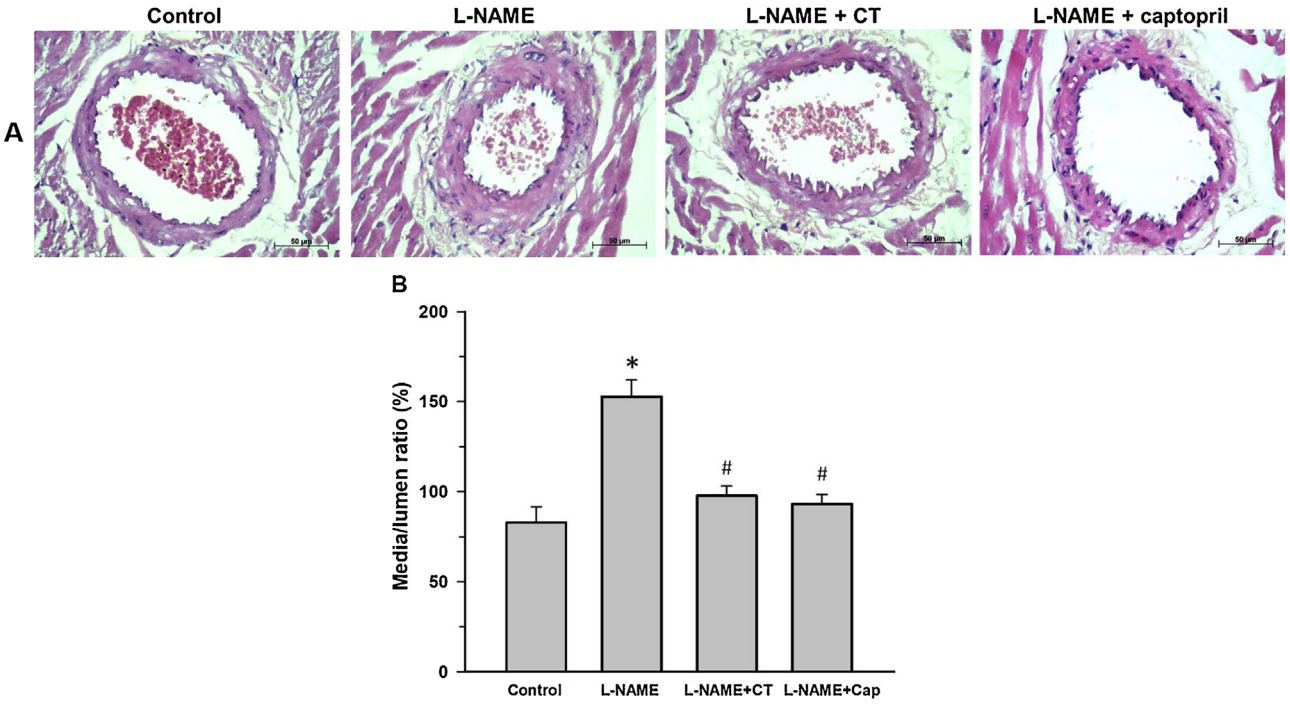

Fig. 3. Effects of CT extract and captopril on intramyocardial coronary artery morphology in L-NAME-induced hypertensive rats. Representative images of intramyocardial coronary artery morphology under the light microscope using a 40× objective lens (A) and values of percentage media/lumen ratios (B). Results are expressed as mean ± SEM. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. L-NAME group ($n = 8$).

heart weight/BW and LVW/BW ratio compared to untreated hypertensive rats ($p < 0.05$).

3.3. *Effect of CT extract and captopril on LV and intramyocardial coronary artery morphology*

Histomorphometric analysis showed that chronic administration of L-NAME significantly increased wall thickness and the cross-sectional area of the LV compared with the control group ($p < 0.05$, Fig. 2B and C). This confirms that L-NAME induces LV hypertrophy in rats. Oral supplementation with CT extract or captopril significantly reduced wall thickness and the cross-sectional area of the LV in L-NAME-induced hypertensive rats ($p < 0.05$). In addition, thickness of the intramyocardial coronary artery wall significantly increased in the L-NAME-treated groups, as indicated by increased media/lumen ratios ($p < 0.05$, Fig. 3B). Concomitant sup-

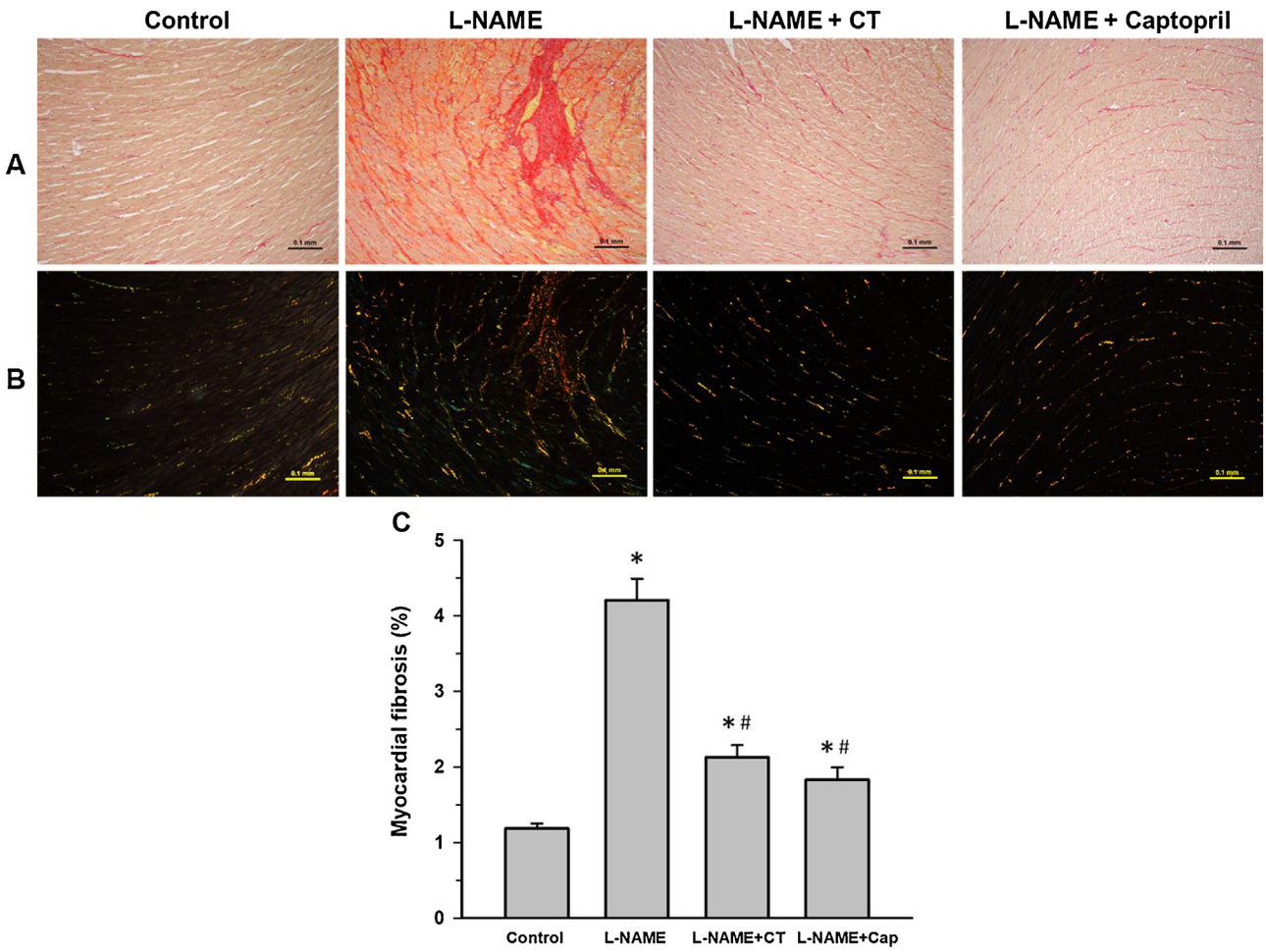

Fig. 4. Effects of CT extract and captopril on myocardial fibrosis in L-NAME-induced hypertensive rats. Representative images of myocardial fibrosis under the light microscope using a 20× objective lens (A), under a polarized light microscope using a 20× objective lens (B) and values of percentage area of myocardial fibrosis (C). Results are expressed as mean ± SEM. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. L-NAME group ($n = 8$).

plementation of L-NAME-treated rats with CT extract or captopril for the last 2 weeks significantly reduced media wall thickness of the intramyocardial coronary artery compared to untreated hypertensive rats ($p < 0.05$).

3.4. Effect of CT extract and captopril on myocardial and perivascular fibrosis

Myocardial interstitial and perivascular fibrosis were assessed from the picrosirius red-stained LV sections by polarized light microscopy. Chronic L-NAME treatment resulted in a significant increase in myocardial fibrosis compared with the control group ($p < 0.05$; Fig. 4C). However, the fibrotic changes induced by L-NAME were attenuated in both the CT extract- and captopril-treated groups compared to the untreated hypertensive group ($p < 0.05$). Moreover, quantitative analysis showed that the percentages of perivascular areas with fibrosis were significantly increased by L-NAME treatment, but that these changes were reduced by CT extract or captopril treatment ($p < 0.05$; Fig. 5C).

3.5. Effect of CT extract and captopril on plasma NOx concentration

Plasma NOx levels from the different experimental groups are shown in Fig. 6. In the L-NAME-treated group, plasma NOx concentration was significantly reduced compared with the control group (3.1 ± 0.5 μmol/L versus 9.9 ± 0.7 μmol/L; $p < 0.05$). Captopril treatment restored plasma NOx concentration to the level of control rats (8.5 ± 0.9 μmol/L), while CT extract improved plasma NOx concentration but to levels still significantly lower than the control rats (6.2 ± 0.6 μmol/L; $p < 0.05$).

3.6. Effect of CT extract and captopril on plasma TGF-β1 concentration

An increase in plasma TGF-β1 level was found in L-NAME-induced hypertensive rats compared to the control rats (26.9 ± 3.4 ng/mL versus 6.5 ± 0.6 ng/mL; $p < 0.05$; Fig. 7). The increase in plasma TGF-β1 level in L-NAME-treated rats was significantly decreased by both CT extract and captopril treatment (8.4 ± 0.4 ng/mL and 6.8 ± 0.9 ng/mL; $p < 0.05$).

3.7. Effect of CT extract and captopril on TGF-β1 and MMP9 protein expression in heart tissue

TGF-β1 and MMP9 protein expression in heart tissue from the different experimental groups are shown in Fig. 8A and B. Upregulation of protein TGF-β1 and protein MMP9 expression was observed in the L-NAME-treated rats ($p < 0.05$). Treatment with CT extract or captopril inhibited TGF-β1 and MMP9 protein overexpression in L-NAME-induced hypertensive rats ($p < 0.05$).

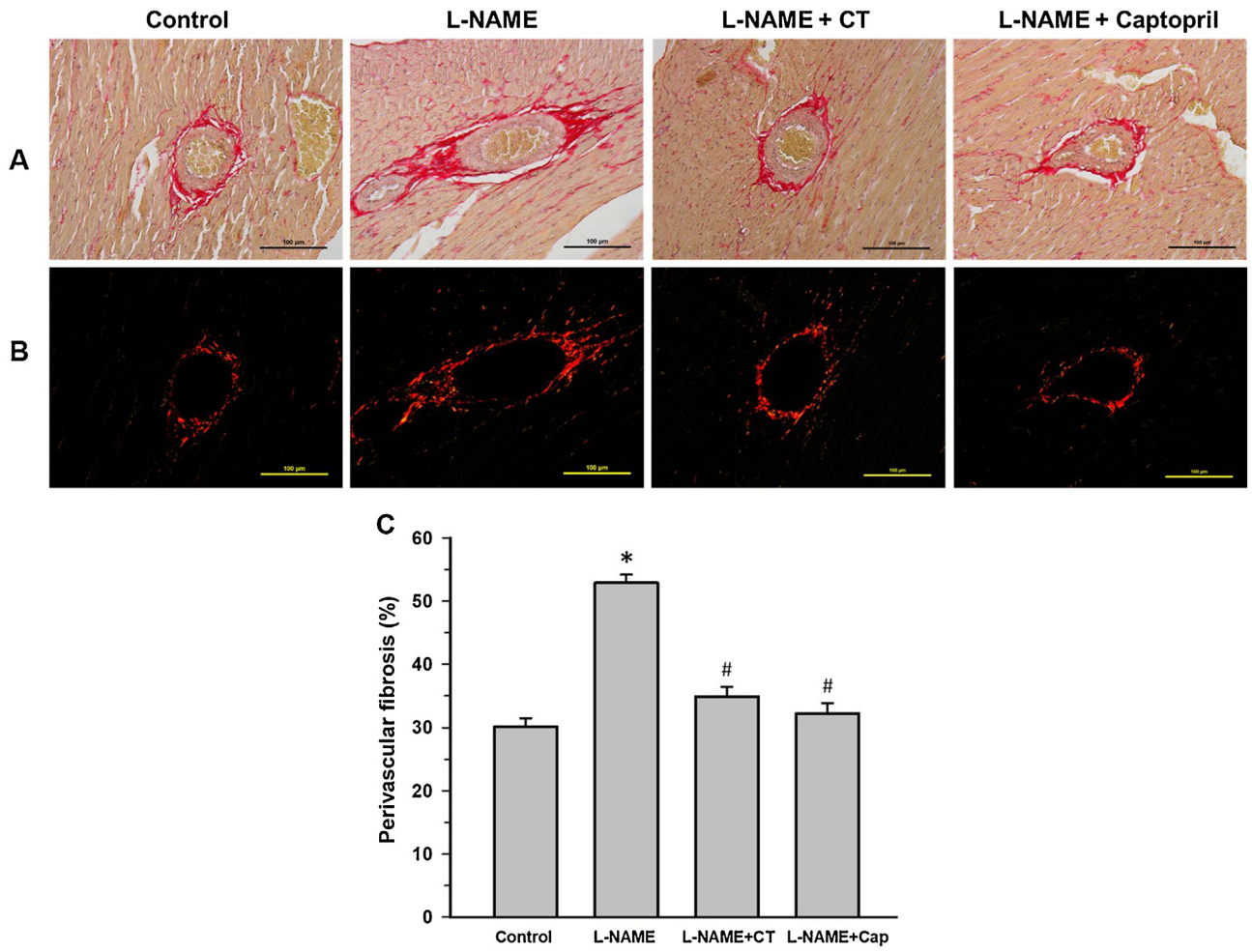

Fig. 5. Effects of CT extract and captopril on perivascular fibrosis in L-NAME-induced hypertensive rats. Representative images of perivascular fibrosis under a light microscope using a 20× objective lens (A), under a polarized light microscope using a 20× objective lens (B) and values of percentage area of perivascular fibrosis (C). Results are expressed as mean ± SEM. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. L-NAME group ($n = 8$).

3.8. Effect of CT extract and captopril on plasma MDA concentration and gp91^{phox} protein expression in heart tissue

The concentration of MDA in plasma was significantly increased in L-NAME-induced hypertensive rats compared to the control rats (9.4 ± 0.6 μmol/L versus 3.3 ± 0.3 μmol/L; $p < 0.05$; Fig. 9A). Moreover, an increase in plasma MDA level was consistent with upregulation of gp91^{phox} protein expression in heart tissue from L-NAME-treated rats ($p < 0.05$; Fig. 9B). Hypertensive rats treated with CT extract or captopril had a significantly reduced plasma MDA level (5.9 ± 0.4 μmol/L and 5.3 ± 0.3 μmol/L; $p < 0.05$) and the overexpression of gp91^{phox} protein in heart tissue was completely suppressed compared to untreated rats ($p < 0.05$).

4. Discussion

In this study, we have demonstrated that CT extract and captopril alleviate L-NAME-induced LV hypertrophy, cardiac interstitial and perivascular fibrosis, and intramyocardial coronary artery remodeling. These therapeutic effects are associated with an increase in plasma NOx level, a decrease in plasma TGF-β1 level, and downregulation of TGF-β1 and MMP-9 protein expression in heart tissue. Furthermore, CT extract and captopril markedly reduced oxidative stress, indicated by a decrease in plasma MDA level and cardiac gp91^{phox} expression.

Our results confirm and extend previous findings that L-NAME induces hypertension and concomitant cardiovascular remodeling, including LV hypertrophy, myocardial interstitial and perivascular fibrosis, and intramyocardial coronary artery remodeling (Boonprom et al., 2017; Sonoda et al., 2017). Systemic hypertension imparts a chronic augmentation of workload on the LV and is the most common cause of LV hypertrophy (Kenchaiah and Pfeffer, 2004). In this current study, administration of L-NAME for 5 weeks increase in blood pressure, representing high afterload that can mediate LV remodeling. Supplementation of CT extract decreased blood pressure in L-NAME hypertensive rats and alleviated changes in LV morphology. The results indicate that treatment with CT extract might result in a lower workload and alleviate cardiac remodeling. However, CT extract was found to partially decrease blood pressure in L-NAME hypertensive rats, and completely normalized cardiac morphological alterations. These findings suggest that it is not just the antihypertensive activity of CT extract, but other effects that contribute to cardioprotection in L-NAME-treated rats.

It is widely known that there are several non-hemodynamic factors, which induce cardiac remodeling in this animal model. Depletion of NO is one non-hemodynamic factor that plays an important role in the development of cardiac remodeling. Previous studies have shown that upregulation of eNOS expression and restoration of NOx levels inhibits cardiac hypertrophy and fibrosis in L-NAME-induced hypertensive rats and mice (Silambarasan

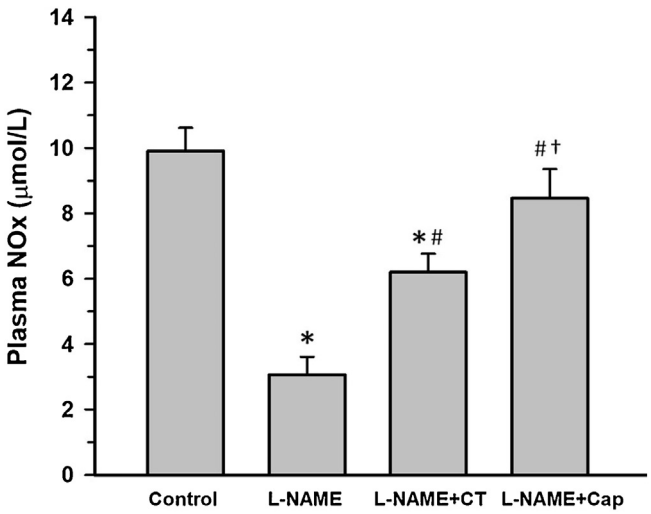

Fig. 6. Effects of CT extract and captopril on plasma NOx concentration in L-NAME-induced hypertensive rats. Results are expressed as mean ± SEM. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. L-NAME group, $^{\dagger}p < 0.05$ vs. L-NAME + CT group ($n = 7$).

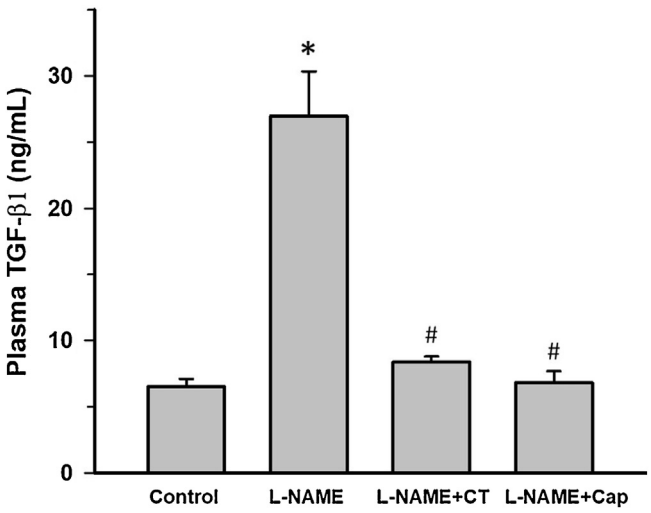

Fig. 7. Effects of CT extract and captopril on plasma TGF-β1 concentration in L-NAME-induced hypertensive rats. Results are expressed as mean ± SEM. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. L-NAME group ($n = 7$).

et al., 2014; Zhang et al., 2012). The current study confirms that treatment of L-NAME-induced hypertensive rats with CT extract reduces blood pressure and restores plasma NOx concentration (Maneesai et al., 2016). In addition, CT extract alleviated cardiac hypertrophy and fibrosis in L-NAME-induced hypertension, possibly due to restoration of plasma NOx concentration. Therefore, our findings indicate that CT extract enhances NO bioavailability, resulting in decreased blood pressure, and diminishes cardiac remodeling in L-NAME hypertensive rats.

TGF-β1, a locally generated cytokine, is implicated as a major mediator of tissue fibrosis (Border and Noble, 1994). It has a major influence on ECM production by upregulating collagen and fibronectin synthesis and by decreasing matrix degradation (Li et al., 2000). In our experimental animals, we confirmed that upregulation of myocardial TGF-b protein expression, increases plasma TGF-β1 concentration and cardiac remodeling (Sonoda et al., 2017). This suggests that overexpression of TGF-β1 may be involved in L-NAME-mediated cardiac morphological alterations. However, treatment with CT extract was effective in decreasing plasma TGF-β1 concentration and expression of TGF-β1 in L-NAME-treated rats. These effects were accompanied by normalization of cardiac hypertrophy and fibrosis. Several previous studies have also reported that inhibition of TGF-β1 expression attenuates myocardial fibrosis and intramyocardial coronary artery remodeling in hypertensive rats (Akashiba et al., 2008; Zhao et al., 2015). Moreover, blockade of TGF-beta receptor 1 alleviated cardiac fibrosis in an experimental mouse model of myocardial infarction (Ellmers et al., 2008). Therefore, the cardioprotective effect of CT extract may be related to its inhibition of TGF-β1-mediated cardiac remodeling.

Increased levels of matrix metalloproteinases, especially MMP-9, mediate collagen breakdown and ECM turnover, leading to fibrosis and LV hypertrophy (Opie et al., 2006). Jiang et al. have shown that MMP-9 inhibition prevents cardiac remodeling in spontaneously hypertensive rats (Jiang et al., 2013). It has also been confirmed that cardiac MMP-9 protein expression increases after L-NAME-induced cardiac morphological abnormalities (Ma et al., 2017). The current study showed that these responses are attenuated by CT treatment. This finding is consistent with a previous study, which showed that CT extract decreases MMP-9 expression and inhibits LPS-induced cardiac fibrosis in rats (Han et al., 2017). Taken together, these results indicate that CT extract inhibits the

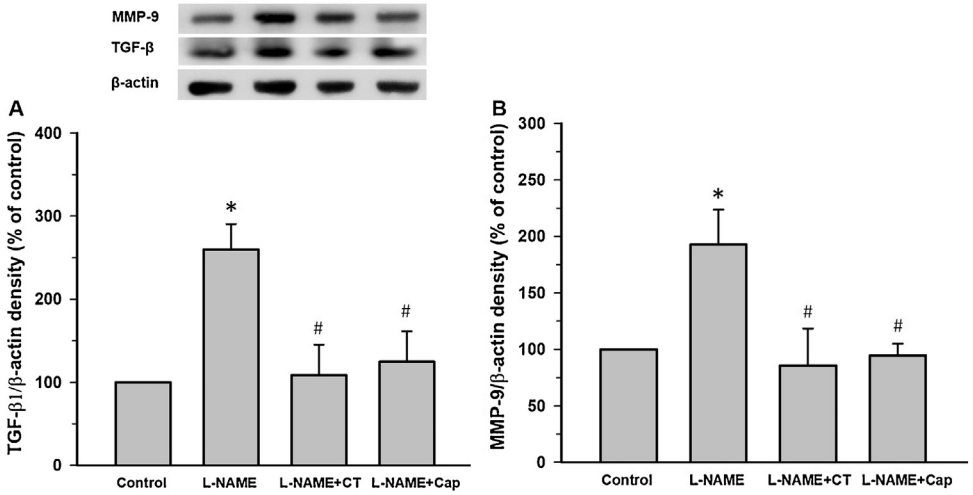

Fig. 8. Effects of CT extract and captopril on TGF-β1 (A) and MMP-9 (B) protein expression from heart tissue in L-NAME-induced hypertensive rats. Results are expressed as mean ± SEM. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. L-NAME group ($n = 4$).

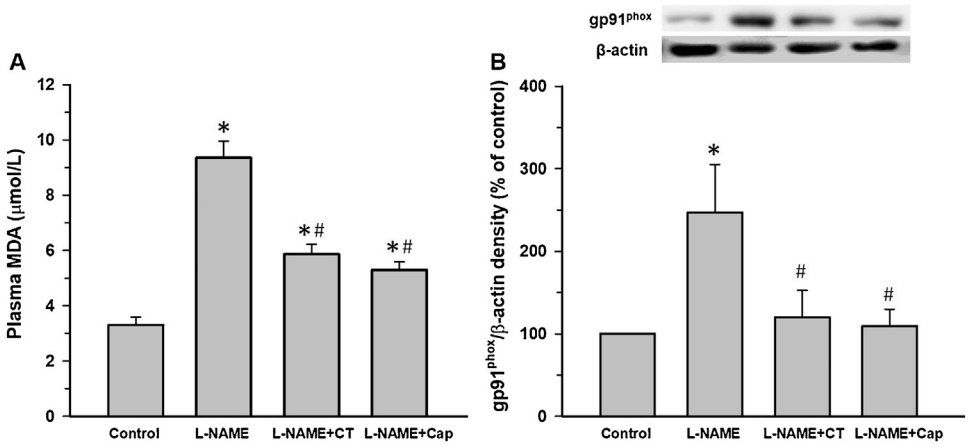

Fig. 9. Effects of CT extract and captopril on plasma MDA concentration (A) and gp91^{phox} protein expression from heart tissue (B) in L-NAME-induced hypertensive rats. Results are expressed as mean ± SEM. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. L-NAME group (plasma MDA $n = 7$; gp91^{phox} protein expression $n = 4$).

cardiac hypertrophy and fibrosis associated with downregulation of cardiac MMP-9 protein expression.

Oxidative stress is also a factor in L-NAME-induced cardiac remodeling. In the current study, it was found that oxidative stress was accompanied by increased myocardium gp91^{phox} expression and an increased plasma MDA level. Recent studies indicate that gp91^{phox} subunit NADPH oxidase-derived ROS play a pivotal role in cardiac pathophysiology. An upregulation of gp91^{phox} expression, the main source of endogenous superoxide production, mediates cardiac fibrosis by enhancing TGF-β1 synthesis in hypertensive rats (Zhao et al., 2008). In addition, ROS derived specifically from gp91^{phox} subunit NADPH oxidase induced cardiac remodeling in mice and was associated with increased MMP-9 activity (Zhao et al., 2010). Our results show that CT extract inhibits the cardiac hypertrophy and fibrosis that accompanies downregulation of cardiac gp91^{phox} expression. This supports previous findings that CT extract suppresses gp91^{phox} expression and alleviates vascular remodeling in renovarcular hypertensive rats (Bunbupha et al., 2018). Thus, the antioxidative activity of CT extract might be partially responsible for the attenuation of cardiac remodeling.

The current study used captopril as a positive control to reduce blood pressure and to alleviate cardiac structural changes in L-NAME-induced hypertensive rats. Our results confirm that captopril reduced blood pressure and effectively alleviated LV hypertrophy and fibrosis in this animal model (Bernatova et al., 1999; Simko et al., 2010). Additionally, captopril 5 mg/kg/day was more effective in reducing blood pressure than CT extract, though both treatments normalized L-NAME-mediated cardiac remodeling. This suggests that non-hemodynamic factors are an important therapeutic target in the prevention of cardiac remodeling.

In conclusion, CT extract reduces blood pressure and alleviates cardiac remodeling in L-NAME-induced hypertensive rats. These effects are likely to be mediated by suppression of the NADPH oxidase-mediated TGF-β1-MMP-9 signaling pathway. These findings support the premise that CT extract could be useful as a complementary medicine in hypertensive heart disease management.

Conflicts of interest

The authors declare no conflict of interest.

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References

Akashiba, A., Ono, H., Ono, Y., Ishimitsu, T., Matsuoka, H., 2008. Valsartan improves L-NAME-exacerbated cardiac fibrosis with TGF-ss inhibition and apoptosis induction in spontaneously hypertensive rats. J. Cardiol. 52, 239–246.

Bernatova, I., Pechanova, O., Simko, F., 1999. Effect of captopril in L-NAME-induced hypertension on the rat myocardium, aorta, brain and kidney. Exp. Physiol. 84, 1095–1105.

Boonprom, P., Boonla, O., Chayaburakul, K., Welbat, J.U., Pannangpetch, P., Kukongviriyapan, U., Kukongviriyapan, V., Pakdeechote, P., Prachaney, P., 2017. *Garcinia mangostana* pericarp extract protects against oxidative stress and cardiovascular remodeling via suppression of p47(phox) and iNOS in nitric oxide deficient rats. Ann. Anat. 212, 27–36.

Border, W.A., Noble, N.A., 1994. Transforming growth factor beta in tissue fibrosis. N. Engl. J. Med. 331, 1286–1292.

Bunbupha, S., Prachaney, P., Kukongviriyapan, U., Kukongviriyapan, V., Welbat, J.U., Pakdeechote, P., 2015. Asiatic acid alleviates cardiovascular remodelling in rats with L-NAME-induced hypertension. Clin. Exp. Pharmacol. Physiol. 42, 1189–1197.

Bunbupha, S., Wunpathe, C., Maneesai, P., Berkban, T., Kukongviriyapan, U., Kukongviriyapan, V., Prachaney, P., Pakdeechote, P., 2018. *Carthamus tinctorius* L. extract improves hemodynamic and vascular alterations in a rat model of renovascular hypertension through Ang II-AT1R-NADPH oxidase pathway. Ann. Anat. 216, 82–89.

Draper, H.H., Squires, E.J., Mahmoodi, H., Wu, J., Agarwal, S., Hadley, M., 1993. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radic. Biol. Med. 15, 353–363.

Ellmers, L.J., Scott, N.J., Medicherla, S., Pilbrow, A.P., Bridgman, P.G., Yandle, T.G., Richards, A.M., Protter, A.A., Cameron, V.A., 2008. Transforming growth factor-beta blockade down-regulates the renin-angiotensin system and modifies cardiac remodeling after myocardial infarction. Endocrinology 149, 5828–5834.

Faler, B.J., Macsata, R.A., Plummer, D., Mishra, L., Sidawy, A.N., 2006. Transforming growth factor-beta and wound healing. Perspect. Vasc. Surg. Endovasc. Ther. 18, 55–62.

Fu, P.K., Pan, T.L., Yang, C.Y., Jeng, K.C., Tang, N.Y., Hsieh, C.L., 2016. *Carthamus tinctorius* L. ameliorates brain injury followed by cerebral ischemia-reperfusion in rats by antioxidative and anti-inflammatory mechanisms. Iran. J. Basic Med. Sci. 19, 1368–1375.

Giugliano, D., 2000. Dietary antioxidants for cardiovascular prevention. Nutr. Metab. Cardiovasc. Dis. 10, 38–44.

Halade, G.V., Jin, Y.F., Lindsey, M.L., 2013. Matrix metalloproteinase (MMP)-9: a proximal biomarker for cardiac remodeling and a distal biomarker for inflammation. Pharmacol. Ther. 139, 32–40.

Han, C.K., Tien, Y.C., Jine-Yuan Hsieh, D., Ho, T.J., Lai, C.H., Yeh, Y.L., Hsuan Day, C., Shen, C.Y., Hsu, H.H., Lin, J.Y., Huang, C.Y., 2017. Attenuation of the LPS-induced, ERK-mediated upregulation of fibrosis-related factors FGF-2, uPA, MMP-2, and MMP-9 by *Carthamus tinctorius* L. in cardiomyoblasts. Environ. Toxicol. 32, 754–763.

Han, S.Y., Li, H.X., Ma, X., Zhang, K., Ma, Z.Z., Tu, P.F., 2009. Protective effects of purified safflower extract on myocardial ischemia in vivo and in vitro. Phytomedicine 16, 694–702.

Hocevar, B.A., Howe, P.H., 2000. Analysis of TGF-beta-mediated synthesis of extracellular matrix components. Methods Mol. Biol. 142, 55–65.

Jiang, B., Li, D., Deng, Y., Teng, F., Chen, J., Xue, S., Kong, X., Luo, C., Shen, X., Jiang, H., Xu, F., Yang, W., Yin, J., Wang, Y., Chen, H., Wu, W., Liu, X., Guo, D.A., 2013. Sal-

vianolic acid A, a novel matrix metalloproteinase-9 inhibitor, prevents cardiac remodeling in spontaneously hypertensive rats. PLoS One 8, e59621.
Kearney, P.M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P.K., He, J., 2005. Global burden of hypertension: analysis of worldwide data. Lancet 365, 217–223.
Kenchaiah, S., Pfeffer, M.A., 2004. Cardiac remodeling in systemic hypertension. Med. Clin. North Am. 88, 115–130.
Kurrelmeyer, K., Kalra, D., Bozkurt, B., Wang, F., Dibbs, Z., Seta, Y., Baumgarten, G., Engle, D., Sivasubramanian, N., Mann, D.L., 1998. Cardiac remodeling as a consequence and cause of progressive heart failure. Clin. Cardiol. 21, I14–19.
Li, Y.Y., McTiernan, C.F., Feldman, A.M., 2000. Interplay of matrix metalloproteinases, tissue inhibitors of metalloproteinases and their regulators in cardiac matrix remodeling. Cardiovasc. Res. 46, 214–224.
Loo, W.T., Cheung, M.N., Chow, L.W., 2004. The inhibitory effect of a herbal formula comprising ginseng and *Carthamus tinctorius* on breast cancer. Life Sci. 76, 191–200.
Ma, J., Ma, S.Y., Ding, C.H., 2017. Curcumin reduces cardiac fibrosis by inhibiting myofibroblast differentiation and decreasing transforming growth factor beta1 and matrix metalloproteinase 9/tissue inhibitor of metalloproteinase 1. Chin. J. Integr. Med. 23, 362–369.
Maneesai, P., Prasarttong, P., Bunbupha, S., Kukongviriyapan, U., Kukongviriyapan, V., Tangsucharit, P., Prachaney, P., Pakdeechote, P., 2016. Synergistic antihypertensive effect of *Carthamus tinctorius* L. extract and captopril in L-NAME-induced hypertensive rats via restoration of eNOS and AT(1)R expression. Nutrients 8, 122.
Opie, L.H., Commerford, P.J., Gersh, B.J., Pfeffer, M.A., 2006. Controversies in ventricular remodelling. Lancet 367, 356–367.
Pechanova, O., 2007. Contribution of captopril thiol group to the prevention of spontaneous hypertension. Physiol. Res. 56 (Suppl. 2), S41–S48.
Pechanova, O., Bernatova, I., Babal, P., Martinez, M.C., Kysela, S., Stvrtina, S., Andriantsitohaina, R., 2004. Red wine polyphenols prevent cardiovascular alterations in L-NAME-induced hypertension. J. Hypertens. 22, 1551–1559.
Sawada, N., Itoh, H., Miyashita, K., Tsujimoto, H., Sone, M., Yamahara, K., Arany, Z.P., Hofmann, F., Nakao, K., 2009. Cyclic GMP kinase and RhoA Ser188 phosphorylation integrate pro- and antifibrotic signals in blood vessels. Mol. Cell Biol. 29, 6018–6032.
Shi, Y.X., Chen, Y., Zhu, Y.Z., Huang, G.Y., Moore, P.K., Huang, S.H., Yao, T., Zhu, Y.C., 2007. Chronic sodium hydrosulfide treatment decreases medial thickening of intramyocardial coronary arterioles, interstitial fibrosis, and ROS production in spontaneously hypertensive rats. Am. J. Physiol. Heart Circ. Physiol. 293, H2093–H2100.
Silambarasan, T., Manivannan, J., Krishna Priya, M., Suganya, N., Chatterjee, S., Raja, B., 2014. Sinapic acid prevents hypertension and cardiovascular remodeling in pharmacological model of nitric oxide inhibited rats. PLoS One 9, e115682.
Simko, F., Pechanova, O., Pelouch, V., Krajcirovicova, K., Celec, P., Palffy, R., Bednarova, K., Vrankova, S., Adamcova, M., Paulis, L., 2010. Continuous light and L-NAME-induced left ventricular remodelling: different protection with melatonin and captopril. J. Hypertens. 28 (Suppl. 1), S13–S18.
Siwik, D.A., Pagano, P.J., Colucci, W.S., 2001. Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts. Am. J. Physiol. Cell Physiol. 280, C53–C60.
Sonoda, K., Ohtake, K., Uchida, H., Ito, J., Uchida, M., Natsume, H., Tamada, H., Kobayashi, J., 2017. Dietary nitrite supplementation attenuates cardiac remodeling in l-NAME-induced hypertensive rats. Nitric Oxide 67, 1–9.
Verdon, C.P., Burton, B.A., Prior, R.L., 1995. Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP+ when the Griess reaction is used to assay for nitrite. Anal. Biochem. 224, 502–508.
Zhang, Y., Cao, Y., Duan, H., Wang, H., He, L., 2012. Imperatorin prevents cardiac hypertrophy and the transition to heart failure via NO-dependent mechanisms in mice. Fitoterapia 83, 60–66.
Zhao, M., Zheng, S., Yang, J., Wu, Y., Ren, Y., Kong, X., Li, W., Xuan, J., 2015. Suppression of TGF-beta1/Smad signaling pathway by sesamin contributes to the attenuation of myocardial fibrosis in spontaneously hypertensive rats. PLoS One 10, e0121312.
Zhao, W., Zhao, T., Chen, Y., Ahokas, R.A., Sun, Y., 2008. Oxidative stress mediates cardiac fibrosis by enhancing transforming growth factor-beta1 in hypertensive rats. Mol. Cell. Biochem. 317, 43–50.
Zhao, Y., McLaughlin, D., Robinson, E., Harvey, A.P., Hookham, M.B., Shah, A.M., McDermott, B.J., Grieve, D.J., 2010. Nox2 NADPH oxidase promotes pathologic cardiac remodeling associated with Doxorubicin chemotherapy. Cancer Res. 70, 9287–9297.
Zhou, X., Tang, L., Xu, Y., Zhou, G., Wang, Z., 2014. Towards a better understanding of medicinal uses of *Carthamus tinctorius* L. in traditional Chinese medicine: a phytochemical and pharmacological review. J. Ethnopharmacol. 151, 27–43.