Ellagic Acid Attenuates Blood Pressure, Oxidative Stress and Increases Aortic Compliance in N^{ω}-nitro-L-arginine Methyl Ester Induced Hypertensive Rats

แอลลาจิค แอซิด ลดความดันเลือด ภาวะเครียดออกซิเดชันและเพิ่มความยืดหยุ่นของหลอดเลือดแดงใหญ่เออร์ตาในหนูขาวความดันเลือดสูงที่ถูกเหนี่ยวนำด้วยสารแอลเนม

Thewarid Berkban (เทวฤทธิ์ เบิกบาน)* Pattanapong Boonprom (พัฒนพงษ์ บุญพรม)**
Sarawoot Bunbupha (สราวุธ บรรบุผา)*** Dr. Parichat Prachaney (ดร.ปาริฉัตร ประจะเนย์)****
Dr.Jariya Umka Welbat (ดร.จริยา อำคา เวลบาท)**** Dr.Poungrat Pakdeechote (ดร.พวงรัตน์ ภักดีโชติ)*****

ABSTRACT

Ellagic acid (EA) is a natural polyphenolic compound and present in several fruits and berries, it has exhibited antioxidant and anti-inflammatory properties. The aimed of the present study was to investigate the protective effect of EA on blood pressure, oxidative stress status and thoracic aorta elastic property in L-NAME induced hypertensive rats. After 5 weeks administration of L-NAME (40 mg/kg) in rats showed significant increases in systolic blood pressure (SBP), vascular superoxide production, plasma malondialdehyde (MDA) and decrease aortic compliance. However, simultaneously treatment with EA (15 mg/kg) for 5 weeks not only significantly attenuated the elevation of SBP, vascular superoxide production, plasma malondialdehyde (MDA) but also increased aortic compliance in hypertensive rats. This study suggests that EA possesses strong antihypertensive and antioxidant properties in L-NAME induced hypertensive rats.

บทคัดย่อ

แอลลาจิค แอซิด คือสารประกอบฟีโนลิคธรรมชาติที่พบในผลไม้และเบอร์รี่หลากหลายชนิด มีคุณสมบัติต้านอนุมูลอิสระและต้านการอักเสบ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลการป้องกันของแอลลาจิค แอซิด ต่อความดันเลือด ภาวะเครียดออกซิเดชั่นและคุณสมบัติความยืดหยุ่นของหลอดเลือดในหนูขาวความดันเลือดสูงที่ถูกเหนี่ยวนำด้วยสารแอลเนม หลังจาก 5 สัปดาห์ในหนูที่ได้รับสารแอลเนม (40 มก./กก.) พบว่ามีการเพิ่มขึ้นของความดันเลือด การผลิต superoxide ในหลอดเลือด malondialdehyde ในเลือด และความยืดหยุ่นของหลอดเลือดลดลง อย่างไรก็ตามการให้แอลลาจิค แอซิด (15 มก./กก.) ร่วมกับ L-NAME เป็นเวลา 5 สัปดาห์ นอกจากสามารถยับยั้งการเพิ่มขึ้นของความดันเลือด การผลิต superoxide ในหลอดเลือด malondialdehyde ในเลือด ยังเพิ่มความยืดหยุ่นของหลอดเลือดในหนูขาวความดันเลือดสูง การศึกษาครั้งนี้แสดงให้เห็นว่าแอลลาจิค แอซิดมีคุณสมบัติลดความดันเลือดและต้านอนุมูลอิสระในหนูขาวความดันเลือดสูงที่ถูกเหนี่ยวนำด้วยสารแอลเนม

Key Words: Hypertension, Ellagic acid, Arterial elasticity
คำสำคัญ: ความดันเลือดสูง แอลลาจิค แอซิด ความยืดหยุ่นของหลอดเลือด

*Student, Doctor of Philosophy in Anatomy, Faculty of Medicine, Khon Kaen University
** Research assistant, Department of Anatomy, Faculty of Medicine, Khon Kaen University
*** Student, Doctor of Philosophy in Physiology, Faculty of Medicine, Khon Kaen University
****Assistant Professor, Department of Anatomy, Faculty of Medicine, Khon Kaen University
*****Assistant Professor, Department of Physiology, Faculty of Medicine, Khon Kaen University

Introduction

Nitric oxide (NO) contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium. NO has been demonstrated and proved to have important role in the maintenance of normal blood pressure (Huang et al., 1995) and body fluid homeostasis (Krier and Romero, 1998). Several disease conditions including essential hypertension have been linked to an impaired synthesis or action of NO (Moncada et al., 1989).

L-arginine analogues are widely used inhibitors of nitric oxide synthase (NOS) activity both in vitro and in vivo in hypertensive model (Kopincova et al., 2012). Administration of NO synthase (NOS) inhibitors like N^{ω}-nitro-L-arginine methyl ester (L-NAME), provides an useful tool for accomplishing NO-deficient conditions (Kopincova et al., 2012), and hence NO biosynthesis, leading to hypertension (Nakmareong et al., 2012; Nyadjeu et al., 2013). Moreover, increased oxidative stress markers such as vascular superoxide ($O_2^{\bullet -}$) production, plasma malondialdehyde (MDA) concentrations and plasma protein carconyl has been reported in L-NAME hypertensive rats (Nakmareong et al., 2012; Priviero et al., 2007; Sung et al., 2013).

Ellagic acid (EA) is a natural polyphenolic compound and present in several fruits and berries such as pomegranates, grapes, strawberries, raspberries, blackberries and walnuts. Previous studies shown that EA has exhibited antioxidant (Murugan et al., 2009), anticancer (Umesalma and Sudhandiran, 2011), antifibrosis (Han et al., 2006) and anti-inflammatory (Corn et al., 2013) activities. However, less is known about protective effects of EA on oxidative stress and aortic elasticity property in L-NAME induced hypertensive rats.

Objectives of the study

Present study was to investigate the protective effect of EA on the arterial blood pressure, oxidative stress status and thoracic elastic property in L-NAME induced hypertensive rats.

Methodology

Animal and experimental protocols

Male Sprague-Dawley rats weighing 240-280 g body weight were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. Rats were housed in stainless steel cages and maintained in an air-conditioned room (25.1±1˚C) with 12 h dark-light cycle at Northeast Laboratory Animal Center. They were fed with a standard chow diet (Chareon Pokapan Co. Ltd., Thailand) and tap water ad libitum. All procedures are complied with the standards for the care and use of experimental animal and approved by Animal Ethics Committee of Khon Kaen University (AEKKU 70/2555).

After seven days acclimatization, the rats were randomly divided into two major experimental groups. Rats in the normal control group received tap water, whereas rats in the L-NAME-treated group received L-NAME (Sigma Chemical Co.) 40 mg/kg/day in their drinking water for 5 weeks. Animals in each group were randomly assigned into two subgroups of eight to ten animals, consisting of (1) the control group receiving distilled water by gavage; (2) control+EA treated group receiving EA at doses of 15 mg/kg/day by gavage; (3) L-NAME group receiving distilled water by gavage; and (4) L-NAME+EA treated group receiving EA at dose of 15

mg/kg/day by gavage for 5 weeks. The doses of EA were chosen on the basis of previous studies in experimental models of animals (Kannan et al., 2011). The body weight was measured every week until the end of experiment.

Blood pressure measurement

Systolic blood pressure (SBP) was monitored and recorded every week in conscious rats by using tail-cuff plethysmography (Blood pressure analyzer, model 29; IITC, Woodland Hills, California, USA) to evaluate the development of the hypertension during EA treatment. The mean values of three measurements were obtained from each rat.

Superoxide ($O_2^{\cdot -}$) production assay

Vascular $O_2^{\cdot -}$ production was measured using lucigenin-enhanced chemiluminescence method as described previously (Luangaram et al., 2007). In short, the carotid arteries were rapidly excised from the animal and cleaned of adherent fat and connective tissue on ice. The vessel segments (3–5 mm) were placed in Krebs-KCl buffer and allowed to equilibrate at 37°C for 30 minutes. Lucien was added in sample tube and placed in luminometer (Turner Biosystems, 23 CA, USA). The photon counts were integrated every 15 second for 5 minutes and averaged. The vessels were dried at 45°C for 24 h, for determination of dry weight. $O_2^{\cdot -}$ production in vascular tissue was expressed as relative light unit counts per minute per milligram of dry tissue weight.

Malondialdehyde (MDA) assay

The concentration of plasma MDA was measured as thiobarbituric acid reactive substances by a spectrophotometric method as previously described (Luangaram et al., 2007). Briefly, at the end of the experiment, rats were sacrificed by over dosage of the pentobarbital-sodium with peritoneal injection. Plasma samples (150 µl) of were reacted with 10% trichloroacetic acid, 5 mM EDTA, 8% sodium dodecylsulfate, 0.5 µg/ml of butylated hydroxyluene. The mixture was incubated for 10 minutes at room temperature, 500 µl of 0.6% thiobarbituric acid was added, and the mixture was boiled in a water bath for 30 minutes. After cooling to room temperature, the mixture was centrifuged for 5 minutes at 10,000 g. The absorbance of the supernatant was measured at 532 nm by spectrophotometer. A standard curve was generated with appropriate concentrations of 1,1,3,3-tetraethoxypropane (0.3–10 µM).

Assessment of aortic elasticity

The elastic properties of the thoracic aorta were measured in situ by diameter changed of vessel, while pressurizing to the vessel. To determined vascular elasticity, the rats were dissected to expose the thoracic aorta. Two polyethylene catheters were inserted into the thoracic aorta, one distal to the arch and the other just below the diaphragm. The normal salines were flush at lower catheter of the thoracic cannula to remove any thrombi, and then a barium sulphate perfusion apparatus was attached at upper catheter of the thoracic cannula. The animals and the instrument were placed under a camera fitted with a macro lens. Initially, the vessel was pressurized with 200 mmHg for 30 seconds in order to approach a preconditioning condition. After deflation, the aorta was extending to pressures of 0, 10, 20, 30, 50, 70, 90, 110, 120, 140, 160, 180, 200, 220 and 240 mmHg for 2 rounds. Each pressure was maintained for 30 seconds to ensure equilibration of pressure between the perfusion instruments. The vascular images of each pressure were captured and processed with image analysis software (Image-Pro Plus, MN, USA) for calculate the external diameter of the vessel. After

completing the inflation experiment, the in-situ length of the thoracic aorta was measured and weighed (Nakmareong et al., 2012).

Statistical analysis

Data was expressed as mean ± S.E.M. Statistical differences were evaluated by one-way analysis of variance (ANOVA) and followed by Student Newman-Keul's test to show specific group differences. All analysis was performed using Sigmastat software version 3.1. Statistical significance was determined at a level of p values<0.05.

Results

The body weight was observed during the experimental period. There were not significant different among groups throughout the experimental (data not shown).

Effect of EA on systolic blood pressure

SBP was measured at stages of pre-treatment (0), 1, 2, 3, 4 and 5 weeks. Before L-NAME administration, the values of SBP were similar among all experimental groups. The values of SBP gradually increased in the first week of L-NAME administration. After five week administration of L-NAME, SBP were significantly increased in L-NAME group (61.45%), and L-NAME+EA 15 mg/kg/day (30.92%) when compared with the baseline of each group. SBP in L-NAME+EA treated group was significantly decreased (18.15%) than those treated with L-NAME alone however, the blood pressure in L-NAME+EA-treated group was still significantly higher than those of the normal controls. Whereas, control+EA treated group maintained a normal blood pressure level throughout the period of the experiment, indicating that the EA do not have a hypotensive effect when administered alone (Figure 1).

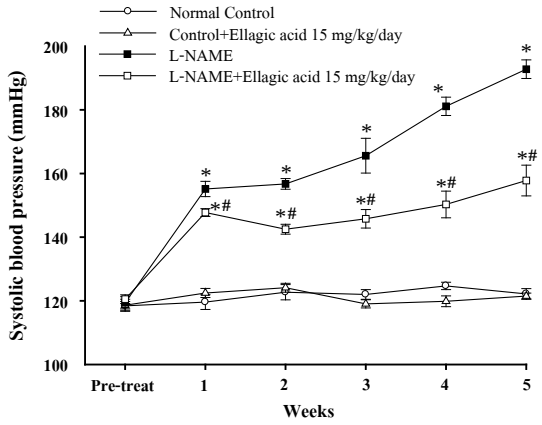

Figure 1 Effect of EA on systolic blood pressure during L-NAME administration for 5 weeks (n=8 per group). *p<0.05 compared with normal control group, #p<0.05 compared with L-NAME group

Effect of EA on superoxide production and plasma malondialdehyde

The values of vascular $O_2^{\cdot -}$ production and plasma MDA of the L-NAME group were significantly increased two-fold and three-fold compared to those in the control group, respectively. However, the vascular $O_2^{\cdot -}$ production and plasma MDA in L-NAME+EA treated group were significantly decreased when compared to rats received L-NAME alone. Moreover, control+EA treated group did not suppress the basal level of vascular $O_2^{\cdot -}$ production and plasma MDA (Figure 2 and 3).

Effect of EA on aortic elasticity

Figures 4a has shown the relationship between pressure and aortic wall thickness. After five week administration of L-NAME, the relative wall thickness (the ratio of the wall thickness to radius) (Figure 4a) were significantly increased in L-NAME group when compared with the controls group at all pressures. These results indicated that the chronic administration of L-NAME 40 mg/kg/day produced the development of vascular structural changes.

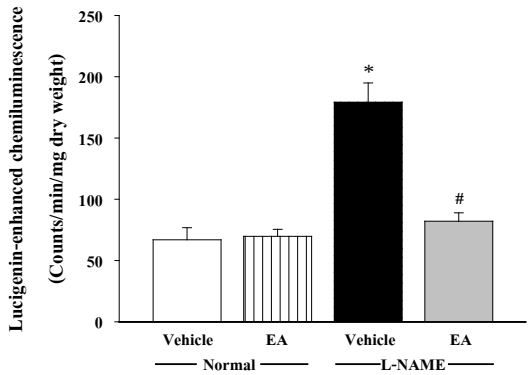

Figure 2 Effect of EA on superoxide production during L-NAME administration for 5 weeks (n=8 per group). *p<0.05 compared with normal control group, #p<0.05 compared with L-NAME group

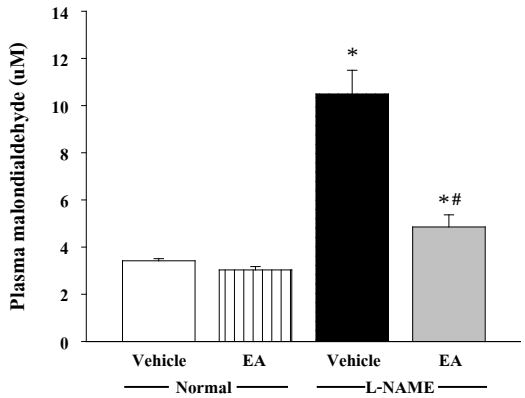

Figure 3 Effect of EA on plasma malondialdehyde during L-NAME administration for 5 weeks (n=7 per group). *p<0.05 compared with normal control group, #p<0.05 compared with L-NAME group

However, the relative wall thickness (Figure 4a) was significantly reduced in L-NAME+EA treated group when compared with the L-NAME group at all pressures.

The mean relative radius (strain) was plotted against all pressure (stress). It was found that the relative radius of the thoracic aorta of L-NAME group was diminished at pressures within the physiological range when compared with the control group, indicating that aortic compliance in the L-NAME group was decreased (Figures 4b). Although, trends of

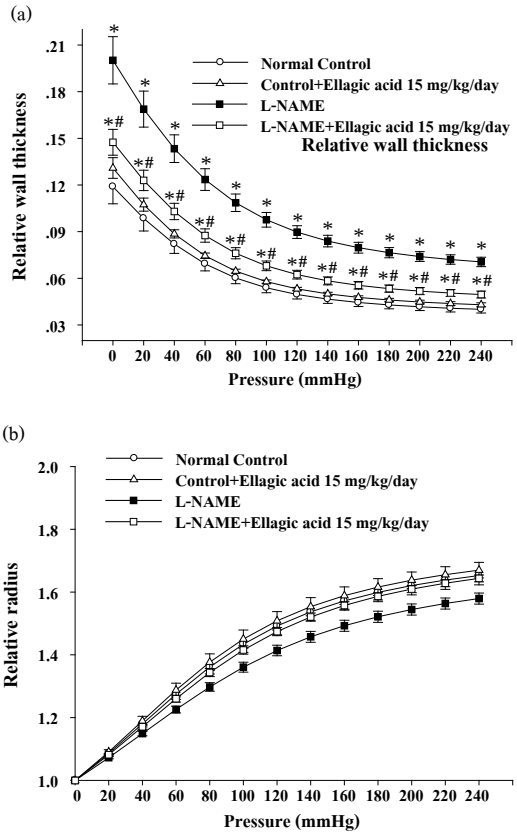

Figure 4 Effect of EA on relative wall thickness (a), relative radius (b) during L-NAME administration for 5 weeks (n=6 per group). *p<0.05 compared with normal control group, #p<0.05 compared with L-NAME group

relative radius values of L-NAME+EA treated group ameliorated the adverse structural remodeling of the aortic wall by the increase in aortic compliance approach to those in control group (Figures 4b), there is no significant difference between groups.

Discussion and Conclusions

This study investigated the protective effect of EA at doses 15 mg/kg/day on blood pressure, oxidative stress status and elasticity property of thoracic aorta in L-NAME induced hypertensive rats. This present study has confirmed that chronic administration of L-NAME 40 mg/kg/day caused a progressive increase in SBP. Moreover, administration of L-NAME for 5 weeks not only increases the blood

pressure but also thoracic aortic stiffness and oxidative stress markers such as vascular superoxide production and plasma malondialdehyde levels.

An elevation in blood pressure was observed within the first week of the treatment. These observations were similar to those obtained by Nyadjeu and coworkers that chronic treatment with L-NAME (40 mg/kg) raised the SBP with associated vascular hypertrophy (Nyadjeu et al., 2013). Likewise, this study demonstrated that L-NAME -induced hypertension significantly decreased in blood pressure when simultaneously treated with the EA at doses 15 mg/kg/day for 5 weeks, indicating that EA has antihypertension property. These observations were similar to study of Mohan and coworkers that chronic treatment with pomegranate juice extracts 100 mg/kg/day and 300 mg/kg/day significantly reduced the elevation of blood pressure in diabetic hypertensive rats induced by angiotensin II (Mohan et al., 2010). In addition, Aviram and coworkers revealed that the patients with carotid artery stenosis reduce the systolic blood pressure after 1 year of pomegranate juice consumption (50 ml/day, which contain 1.5 mmoles of total polyphenols) by 21% (Aviram et al., 2004).

A possible mechanism that described the effect of EA on anti-hypertension was proposed by Chang (2013) and Yilmaz (2012). They demonstrated that treatment with EA inhibited the phosphorylation of phospholipase C (PLC), indicating that EA induces vasorelaxation through direct inhibition of Ca^{2+} mobilization and extracellular Ca^{2+} influx (Chang et al., 2013; Yilmaz and Usta, 2012).

L-NAME treatment induced a vascular stiffness accompanied by increased aortic cross-sectional area (Bernatova et al., 2002). It was well known that the vascular remodeling might be influenced by an imbalance between oxidative stress and antioxidant. This over production of $O_2^{\cdot -}$ may be due to angiotensin II stimulate the NADPH oxidase system to increase reactive oxygen species (ROS) in vascular cells (Nickenig and Harrison, 2002), leading to a promotion of promotes vascular cell growth, inflammation and increase extracellular matrix fibers deposition (Xu and Touyz, 2006). Present study revealed chronic administration of L-NAME 40 mg/kg/day raised the $O_2^{\cdot -}$ production in the arterial tissues, increased plasma MDA and aortic stiffness. These results were consistent with previous studies that chronic blockade of NO synthesis by L-NAME-mediated NOS inhibition leads to increased accumulation of $O_2^{\cdot -}$ generation, increased MDA concentrations, and activation of NADPH oxidase in the heart and aorta (Chang et al., 2013; Nakmareong et al., 2011). Moreover, our study have shown that EA significantly suppressed the levels of $O_2^{\cdot -}$ in the arterial tissues, plasma MDA and enhance aortic compliance in hypertensive rats, suggesting that EA displays antioxidant property. It has been reported that EA (25 and 50 mg/kg BW) significantly protected the liver by restoring the activity of superoxide dismutase, catalase and liver glutathione, and thiobarbturic acid reactive substances with respect to the carbon tetrachloride treated group (Murugan et al., 2009). Moreover, pomegranate juice (100 and 300 µg/kg/day) treatment also caused a significant increase activity of enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GSH) while the levels of thiobarbituric acid reactive substances (TBARS) in kidney and pancreas significant decrease in diabetic hypertensive rats induced by angiotensin II (Mohan et al., 2010). In addition, Aviram and

coworkers demonstrated that pomegranate juice consumption (50 ml/day, which contain 1.5 mmoles of total polyphenols) was significantly reduced common carotid intima-media thickness by up to 30% after 1 year in the patients with carotid artery stenosis (Aviram et al., 2004)

In conclusion, this study revealed that the EA (15 mg/kg/day) possesses strong antihypertensive and antioxidant properties in L-NAME induced hypertensive rats as evidenced by significant decrease in the blood pressure, oxidative stress markers and increase aortic compliance.

Acknowledgements

Thewarid Berkban was supported by a granted from Faculty of Medicine, Khon Kaen University, Thailand.

References

Aviram M, Rosenblat M, Gaitini D, Nitecki S, Hoffman A, Dornfeld L, et al. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. *Clin Nutr* 2004; 23: 423-33.

Bernatova I, Pechanova O, Babal P, Kysela S, Stvrtina S, Andriantsitohaina R. Wine polyphenols improve cardiovascular remodeling and vascular function in NO-deficient hypertension. *Am J Physiol Heart Circ Physiol* 2002; 282: H942-8.

Chang Y, Chen WF, Lin KH, Hsieh CY, Chou DS, Lin LJ, et al. Novel bioactivity of ellagic Acid in inhibiting human platelet activation. *Evid Based Complement Alternat Med* 2013; 2013: 595128.

Corn, xe, lio Favarin D, Martins Teixeira M, Lemos de Andrade E, ia, et al. Anti-Inflammatory Effects of Ellagic Acid on Acute Lung Injury Induced by Acid in Mice. *Mediators of Inflammation* 2013; 2013: 13.

Han DH, Lee MJ, Kim JH. Antioxidant and apoptosis-inducing activities of ellagic acid. *Anticancer Res* 2006; 26: 3601-6.

Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 1995; 377: 239-42.

Kopincova J, Puzserova A, Bernatova I. L-NAME in the cardiovascular system - nitric oxide synthase activator? *Pharmacol Rep* 2012; 64: 511-20.

Krier JD, Romero JC. Systemic inhibition of nitric oxide and prostaglandins in volume-induced natriuresis and hypertension. *Am J Physiol* 1998; 274: R175-80.

Luangaram S, Kukongviriyapan U, Pakdeechote P, Kukongviriyapan V, Pannangpetch P. Protective effects of quercetin against phenylhydrazine-induced vascular dysfunction and oxidative stress in rats. *Food Chem Toxicol* 2007; 45: 448-55.

Mohan M, Waghulde H, Kasture S. Effect of pomegranate juice on Angiotensin II-induced hypertension in diabetic Wistar rats. *Phytother Res* 2010; 24 Suppl 2: S196-203.

Moncada S, Palmer RM, Higgs EA. Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem Pharmacol* 1989; 38: 1709-15.

Murugan V, Mukherjee K, Maiti K, Mukherjee PK. Enhanced oral bioavailability and antioxidant profile of ellagic acid by phospholipids. *J Agric Food Chem* 2009; 57: 4559-65.

Nakmareong S, Kukongviriyapan U, Pakdeechote P, Donpunha W, Kukongviriyapan V, Kongyingyoes B, et al. Antioxidant and vascular protective effects of curcumin and tetrahydrocurcumin in rats with L-NAME-induced hypertension. *Naunyn Schmiedebergs Arch Pharmacol* 2011; 383: 519-29.

Nakmareong S, Kukongviriyapan U, Pakdeechote P, Kukongviriyapan V, Kongyingyoes B, Donpunha W, et al. Tetrahydrocurcumin alleviates hypertension, aortic stiffening and oxidative stress in rats with nitric oxide deficiency. *Hypertens Res* 2012; 35: 418-25.

Nickenig G, Harrison DG. The AT(1)-type angiotensin receptor in oxidative stress and atherogenesis: part I: oxidative stress and atherogenesis. *Circulation* 2002; 105: 393-6.

Nyadjeu P, Nguelefack-Mbuyo EP, Atsamo AD, Nguelefack TB, Dongmo AB, Kamanyi A. Acute and chronic antihypertensive effects of Cinnamomum zeylanicum stem bark methanol extract in L-NAME-induced hypertensive rats. *BMC Complementary and Alternative Medicine* 2013; 13: 27.

Priviero FB, Teixeira CE, Claudino MA, De Nucci G, Zanesco A, Antunes E. Vascular effects of long-term propranolol administration after chronic nitric oxide blockade. *Eur J Pharmacol* 2007; 571: 189-96.

Sung JH, Jo YS, Kim SJ, Ryu JS, Kim MC, Ko HJ, et al. Effect of Lutein on L-NAME-Induced Hypertensive Rats. *Korean J Physiol Pharmacol* 2013; 17: 339-45.

Umesalma S, Sudhandiran G. Ellagic acid prevents rat colon carcinogenesis induced by 1, 2 dimethyl hydrazine through inhibition of AKT-phosphoinositide-3 kinase pathway. *Eur J Pharmacol* 2011; 660: 249-58.

Xu S, Touyz RM. Reactive oxygen species and vascular remodelling in hypertension: still alive. *Can J Cardiol* 2006; 22: 947-51.

Yilmaz B, Usta C. Ellagic acid-induced endothelium-dependent and endothelium-independent vasorelaxation in rat thoracic aortic rings and the underlying mechanism. *Phytother Res* 2012; 27: 285-9.