Natural Product Sciences
20(3) : 216-225 (2014)

Ethylacetate Fraction of *Rubus coreanum* Causes Vascular Relaxation and Hypotensive Action†

Soon-Pyo Hong[1], Mee-Sung Choi[2], and Dong-Yoon Lim[3,*]

[1]Department of Internal Medicine (Cardiology), College of Medicine, Chosun University, Gwangju 501-759, Republic of Korea
[2]Department of Leisure & Sport, College of Public Health & Welfare, Dongshin University, Naju 520-714, Chollanam-Do Province, Republic of Korea
[3]Department of Pharmacology, College of Medicine, Chosun University, Gwangju 501-759, Republic of Korea

Abstract – The present study was designed to investigate whether ethylacetate (EtOAc) fraction extracted from *Rubus coreanum* affect the contractility of the isolated thoracic aortic strips and blood pressure of normotensive rats. The EtOAc fraction (400 μg/mL) significantly depressed both phenylephrine (PE, 10 μM)- and high K^+ (56 mM)-induced contractile responses of the isolated thoracic aortic strips in a concentration-dependent fashion. In the simultaneous presence of L-NAME (an inhibitor of NO synthase, 300 μM) and EtOAc (400 μg/mL), both PE- and high K^+-induced contractile responses were recovered to the corresponding control level in comparison with inhibition of EtOAc-treatment alone. Moreover, in the simultaneous presence of EtOAc after pretreatment with 0.4% CHAPS, both PE- and high K^+-induced contractile responses were recovered to the corresponding control level compared to the inhibitory response of EtOAc-treatment alone. Also, in anesthetized rats, EtOAc fraction (0.3~3.0 mg/kg) injected into a femoral vein dose-dependently produced depressor responses. This hypotensive action of EtOAc fraction was greatly inhibited after treatment with phentolamine (1 mg/kg), chlorisondamine (1 mg/kg), L-NAME (3 mg/kg/30 min) or sodium nitroprusside (30 μg/kg/30 min). Intravenous infusion of EtOAc fraction (1.0~10.0 mg/kg/30 min) markedly inhibited norepinehrine-induced pressor responses. Taken together, these results demostrate that EtOAc causes vascular relaxation in the isolated rat thoracic aortic strips as well as hypotensive action in anesthetized rats. These vasorelaxation and hypotension of EtOAc seem to be mediated at least by the increased NO production through the activation of NO synthase of vascular endothelium, and the inhibitory adrenergic modulation.
Keywords – *Rubus coreanum* (Bokboonja), Ethylacetate (EtOAc) fraction, Vasorelaxation, Hypotension, Adrenergic α_1-receptors blockade, Activation of NO synthase

Introduction

Rubus coreanum MIQUEL has been presently used in treating the disease of the aged, spermatorrhea and impotence in oriental medicine. It is also the principal products of Gochang county, Chonbuk province, Korea, where is famous for wine brewed from *Rubus coreanum* MIQUEL (Bokboonja liquor). So far *Rubus coreanum* has been found to possess several polyphenolic compounds, such as (−)-epicatechin, (+)-catechin, proanthocyanidin, etc. Ethanol extract of *Rubus coreanum* showed the antioxidative activity with inhibitory effects on linoleic acid oxidation and LDL oxidation.[1]

Cho found that total phenol content of extract from *Rubus coreanum* M. was contained highly in hot-water extract than other extracts. These extracts elicited antioxidant protection as well as inhibitory activities on xanthine oxidase, pancreatin, α-amylase, and angiotensin converting enzyme.[2] Previously, it has been demonstrated that polyphenol compounds (PCRC), isolated from Bokboonja liquor, inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats[3] and spontaneously hypertensive rats.[4] It seems that this inhibitory effect of PCRC is exerted by inhibiting both the Ca^{2+} influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the

*Author for correspondence
Dong-Yoon Lim, Professor, Department of Pharmacology, College of Medicine, Chosun University, Gwangju 501-759, Republic of Korea
Tel: +82-62-230-6335; E-mail: dylim@chosun.ac.kr

†This paper was presented at the 23rd Annual Congress of the Korean Society of Hypertension held in Seoul, Korea, May 9-10, 2014.

cytoplasmic calcium store partly through the increased NO production due to the activation of nitric oxide synthase.[3,4]

Several investigators have reported that extracts from grapes and wine induce endothelium-dependent relaxation via enhanced generation and/or increased biological activity of NO leading to the elevation of cGMP levels.[5] The critical step for the activation of NO synthase in endothelial cells is the increase in Ca^{2+} concentration leading to the production of NO and the subsequent endothelium-dependent vasorelaxation.[6] The biological activity of NO can be effectively increased by the scavengers of oxygen-free radicals.[7]

As aforementioned, there are many reports about the effects of red wine on cardiovascular system. Despite of these studies, there are so far few reports on *in vitro* functional effects of fractions isolated from Bokboonja wine on the cardiovascular system. Therefore, the aim of the present study was to investigate the ability of some fractions isolated from Bokboonja wine on the blood pressure, and the contractility of the thoracic aorta isolated from normotensive and spontaneously hypertensive rats, and to clarify its mechanism of action in order to supply information for isolation active antihypertensive components.

Experimental

Fractionation of *Rubus coreanum* – Fractionation of *Rubus coreanum* extract was made from a 1-year old wine brewed from *Rubus coreanum* Miquel at the Research Institute of Bokboonja, Gochang County, Cheollabukdo Province, Korea as shown in Fig. 1 (Upper): wine of *Rubus coreanum* was concentrated in a vacuum. And then it was extracted with methylene chloride (CH_2Cl_2) followed by extraction with ethylacetate (EtOAc) and n-butanol. These fractions were concentrated by vacuum, evaporation and atomized, lyophilized by freezing dryer (Coldvac −80, Hanil R & D, Korea). Extract of 2.10 g CH_2Cl_2, 10.97 g EtOAc and 9.06 g n-butanol was obtained from 6 L Bokboonja wine, respectively. The working solution of these extracts was prepared by dissolving in 0.9% NaCl solution or DMSO on the day of each experiment and filtered before administration and diluted appropriately with Krebs-bicarbonate solution (final concentration of alcohol was less than 0.1 %).

Vasorelaxation – Mature male Sprague-Dowley rats (purchased from DAMOOL SCIENCE, International Customer Service, Seoul, Korea), weighing 200 to 300 grams, were used in the experiment. The animals were housed individually in separate cages, and food (Cheil

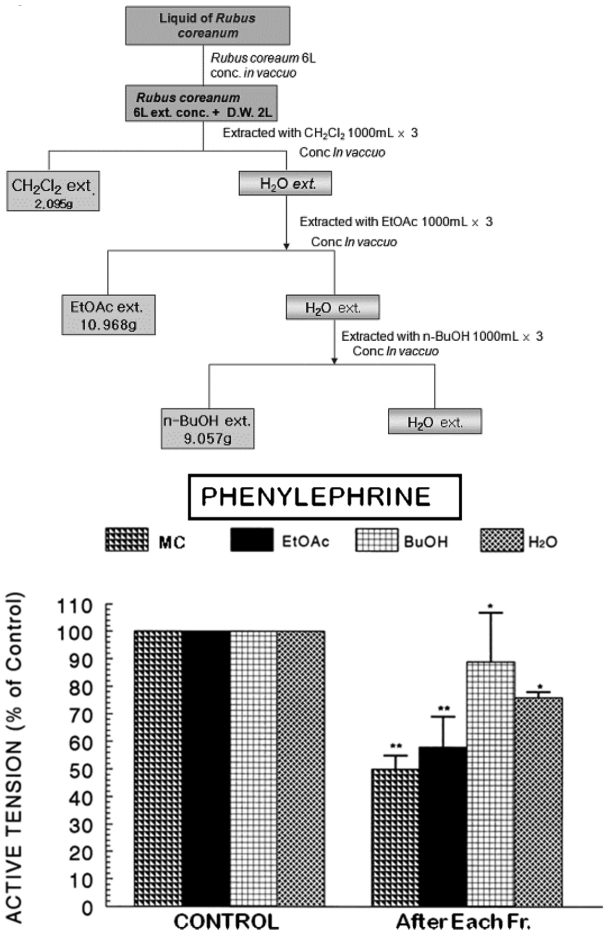

Fig. 1. Fractionation procedure of *Rubus coreanum* (Upper) and comparative effects of four fractions (water [H_2O], butanol [BuOH], ethylacetate [EtOAc], and methylene chloride [MC]) extracted from *Rubus coreanum* on the inhibition of phenylephrine-induced contractile responses in the isolated thoracic aortic strips of rats (Lower). The contractile responses were induced by adding 10 μM PE at 120 min interval after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol. Each column denotes active tension induced evoked by 10 μM PE before and after adding fractions (400 μg/ml) of MC, EtOAc, BuOH and H_2O, respectively. Vertical bars represent the standard error of the mean (S.E.M). Ordinate: the active tension (% of control, 1.6 ± 0.1 g [10 μM]). Abscissa: after treatment of each fraction. Statistical difference was obtained by comparing its control with each fraction-pretreated group. *: $P < 0.05$, **: $P < 0.01$.

Animal Chow) and tap water were allowed *ad libitum* for at least a week to adapt to experimental circumstances. On the day of experiment, a rat was anesthetized with thiopental sodium (50 mg/kg) intraperitoneally, and tied in supine position on fixing panel.

Isolation of thoracic aortic strips – The thorax was opened by a midline incision, and the heart and surrounding area were exposed by placing three hook retractors. The heart and portion of the lung were not removed, but pushed over to the right side and covered by

saline-soaked gauge pads in order to obtain enough working space for isolating thoracic aortic vessel. The aorta was isolated from the proximal part of the heart to the vicinity of liver and immediately immersed in cold Krebs solution. The blood within the aorta was rapidly removed. The aorta was cut into the ring of 4 - 5 mm length.

Recording of mechanical activity – The ring segment of aorta was mounted in a muscle bath by sliding the ring over two parallel stainless-steel hooks (0.15 mm in diameter). The lower hook was fixed on bottom of the bath and the upper was connected to isometric transducer (Grass FT. 03). The signal from the transducer was displayed on a polygraph (Grass Instruments Model 79). The volume of bath was 25 ml and the bath solution was saturated with 95% O_2 and 5% CO_2 at 37 ºC. The composition (mM) of Krebs was: NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.7. The final pH of the solution was maintained at 7.4 - 7.5. During equilibration period of 2 hours, the resting tension was adjusted to 0.5 g. After the equilibration period, the ring was challenged with 35 mM KCl two times, and if it responded with contraction, the proper experiment was started. Vasoconstrictors were administered into the bath in order to obtain dose-response curves. In the subsequent experiments, under the presence of extracts of *Rubus coreanum*, some vasoconstrictors were administered, respectively. The data were expressed as % of the control tension.

Removal of endothelium – A solution containing 0.4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) was perfused for 30 seconds to remove the endothelium,[8] followed by washout with the drug-free solution. The effect of CHAPS was confirmed by the absence of a flow increase due to 10^{-6} M acetylcholine and the presence of a response to 10^{-6} M sodium nitroprusside before the experiments were started. The vasoconstrictor-induced response of non-treated (control) and CHAPS-treated preparations was compared in parallel.

Preparation for measurement of arterial pressure – The animal was tied in supine position on fixing panel to insert a T- formed cannula into the tachea for securing free air passage. The rectal temperature was maintained at 37 - 38 ºC by a thermostatically controlling blanket and heating lamp throughout the course of the experiment.

Measurement of blood pressure – In order to observe the change of arterial pressure, one of the common carotid arteries or of the femoral arteries was catheterized with polyethylene tubing [outside diameter (o.d): 0.5 mm]. The tubing was connected to a pressure transducer (Gould Co., U.S.A.) and pulse of mean arterial blood pressure was recorded on a biological polygraph (Grass Co., U.S.A.) continuously. The chart speed was adjusted to 2 cm per minute. The artery tubing was filled with heparin solution (400 I.U.) to prevent the blood coagulation during the experiment. Another cannulation with polyethylene tubing (o.d.: 0.3 mm) was made into a femoral vein for the administration of drugs and supplemental anesthetic agents as needed to maintain light surgical anesthesia. Each rat was left undisturbed for at least 30 minutes after completion of the operative procedures to permit cardiovascular parameters to be stabilized and drugs under investigation were administered at intervals of 60 minutes.

Statistical analysis – The statistical difference between the control and the pretreated groups was determined by the Student's *t* and ANOVA tests. A P-value of less than 0.05 was considered to represent statistically significant changes unless specifically noted in the text. Values given in the text refer to means and the standard errors of the mean (S.E.M.). The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray.[9]

Drugs and their sources – The following drugs were used: phenylephrine hydrochloride, potassium chloride, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), acetylcholine chloride, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), norepinephrine bitartrate, veratridine chloride, cyclopiazonic acid, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (BAY-K-8644), (Sigma Chemical Co., U.S.A.), chlorisondamine chloride, phentolamine mesylate (CIBA Co., U.S.A.), thiopental sodium and heparin sodium (Daehan Choongwae Pharm. Co., Korea), and 3-(m-cholro-phenyl- carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride [McN-A-343] (RBI, U.S.A.). Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except Bay-K-8644 and CH_2Cl_2 fraction, which were dissolved in 99.5% ethanol and diluted appropriately with Krebs-bicarbonate solution (final concentration of alcohol was less than 0.1%). Concentrations of all drugs except EtOAc fraction used were expressed in terms of molar base.

Results

Effects of four fractions extracted from *Rubus coreanum* on phenylephrine-induced contractile responses in the thoracic aortic strips of normotensive rats – The resting (basal) tension from the isolated rat

aortic strips with intact endothelium reaches a steady state after the perfusion with oxygenated Krebs-bicarbonate solution for 90 min before the experimental protocol is initiated. The resting tension was adjusted to 0.5 g. The effects of four fractions extracted from *Rubus coreanum* on phenylephrine (PE)-induced contractile responses in the rat aorta with intact endothelium were examined. In the present study, the EtOAc fraction itself did not produce any effect on the resting tension in the aortic strips with intact endothelium isolated from rats (data not shown). In previous study, it has been found that PCRC causes vascular relaxation in the isolated aortic strips of SHRs at least partly by the increased NO production through the activation of NO synthase of vascular endothelium, but not through the activation of cyclooxygenase.[10] Therefore, it was attempted to examine effects of four fractions (ethylacetate [EtOAc], methylene chloride [MC], n-butanol [BuOH], and water [H_2O]) isolated from *Rubus coreanum M.* on PE-induced contractile responses in the isolated rat aortic strips. As shown in Fig. 1 (Lower), in the presence of MC (400 μg/mL), EtOAc (400 μg/mL), BuOH (400 μg/mL), and H_2O (400 μg/mL) 5 min before addition of phenylephrine, the contractile responses of phenylephrine (10^{-5} M) were significantly reduced to 50 ± 1% ($P < 0.01$, n = 6), 58 ± 11% ($P < 0.01$, n = 6), 90 ± 18% ($P < 0.01$, n = 8), and 78 ± 2% ($P < 0.05$, n = 6) of the corresponding control response (1.6 ± 0.1 g), respectively. Based on these results, for the PE-induced contractile response, the following rank order of inhibitory potency was obtained: MC > EtOAc >> H_2O >> BuOH. Therefore, in all subsequent experiments, EtOAc fraction (400 μg/ml) only was used.

Effects of the EtOAc fraction isolated from Rubus coreanum on contractile responses induced by phenylephrine and high K^+ in the thoracic aortic strips of normotensive rats and SHRs – Phenylephrine is a selective agonist of adrenergic α_1 receptors, which exhibits vasoconstriction. To establish the inhibitory effect of EtOAc fraction on phenylephrine (10^{-5} M)-induced contractile responses, in the presence of EtOAc fraction at 400 μg/ml, 5 min before addition of phenylephrine, the contractile response of phenylephrine (10^{-5} M) was dose-dependently reduced to 58 ± 11% ($P < 0.01$, n = 10) of the corresponding control response (1.9 ± 0.2 g) (Fig. 2). High K^+ exerts two distinct effects on cells: (1) depolarization of cell membrane, and (2) depolarization- induced influx of calcium via voltage-dependent calcium channels.[11] When added through the bath, high potassium at 5.6×10^{-2} M, which is a membrane-depolarizing agent, caused an increase in aortic contraction (1.3 ± 0.2 g). As shown in Fig. 2, high potassium (5.6×10^{-2} M)-induced contractile response after pre-loading with 400 μg/ml of EtOAc fraction 5 min before high potassium was dose-dependently reduced to 62 ± 8% ($P < 0.01$, n = 10) of the corresponding control response (1.3 ± 0.2 g).

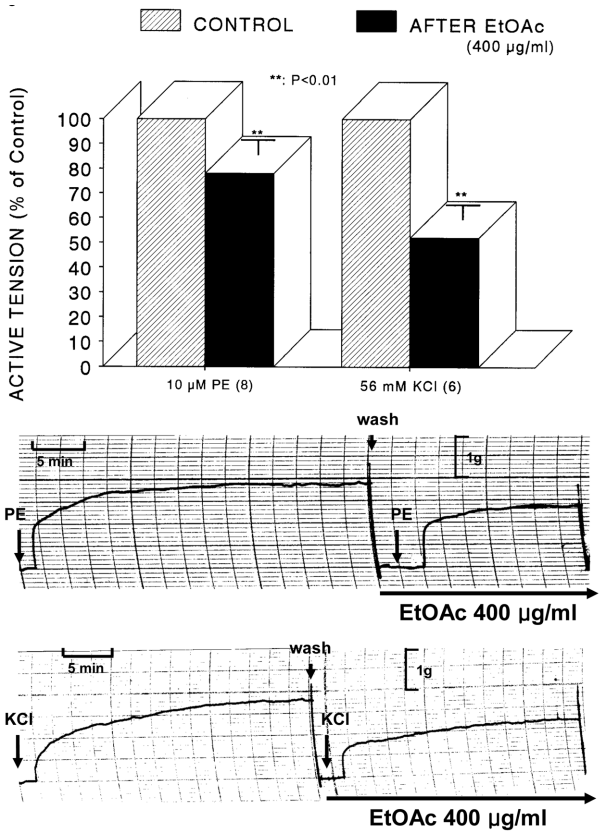

Fig. 2. Effects of EtOAc fraction on phenylephrine (PE)- and high potassium (KCl)-induced contractile responses (Upper) and the typical tracing showing the effect of EtOAc fraction on phenylephrine (PE)- and high potassium (KCl)-induced contractile response in the isolated rat aortic strip (Lower). "Black column" and "Brick column" denote active tension induced evoked by 10 μM PE before and after adding 400 μg/ml of EtOAc fraction, respectively. Other legends and methods are the same as in Fig. 1. **: $P < 0.01$. **[Upper panel]** Left: PE-induced contractile response (Control). Right: PE-induced contractile response in the presence of EtOAc (400 μg/ml). **[Lower panel]** Left: KCl-induced contractile response (Control). Right: KCl-induced contractile response in the presence of EtOAc (400 μg/ml). At arrow mark, the indicated dose of PE (10 μM) and KCl (56 mM) was added into the bath, respectively. The chart speed was 5 mm/min.

Influence of the EtOAc fraction plus L-NAME on the contractile responses evoked by phenylephrine and high potassium in the thoracic aortic strips of normotensive rat – In previous study, it has been demonstrated that PCRC inhibits the CA secretion evoked by cholinergic stimulation and direct membrane-depolarization from the perfused adrenal medulla of SHRs, which was blocked in the presence of L-NAME, a NO synthase

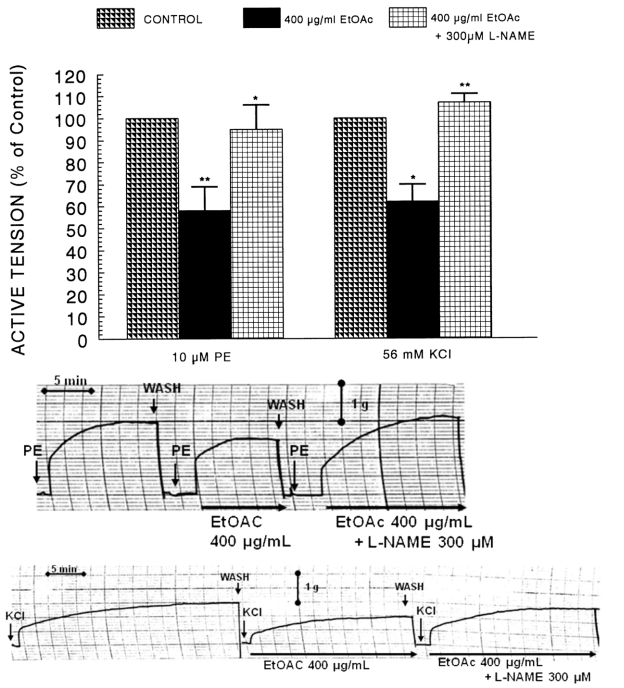

Fig. 3. Influence of EtOAc fraction plus L-NAME on the contractile responses evoked by phenylephrine (PE) and high potassium (KCl) (Upper) and the typical tracing showing the effect of EtOAc fraction plus L-NAME on phenylephrine (PE, upper pannel)- and high potassium (lower pannel)-induced contractile response in the isolated rat aortic strips (Lower). Statistical difference was obtained by comparing the control with the EtOAc fraction-pretreated group or EtOAc fraction (400 μg/ml) plus L-NAME (300 μM). Other legends are the same as in Fig. 2. *: $P < 0.05$, **: $P < 0.01$. **(Upper tracing)**; Left: PE-induced contractile response (Control). Middle: PE-induced contractile response in the presence of EtOAc fraction (400 μg/mL). Right: PE-induced contractile response in the presence of EtOAc fraction (400 μg/mL) plus L-NAME (300 μM). **(Lower tracing)**; Left: KCl-induced contractile response (Control). Middle: KCl-induced contractile response in the presence of EtOAc fraction (400 μg/mL). Right: KCl-induced contractile response in the presence of EtOAc fraction (400 μg/mL) plus L-NAME (300 μM).

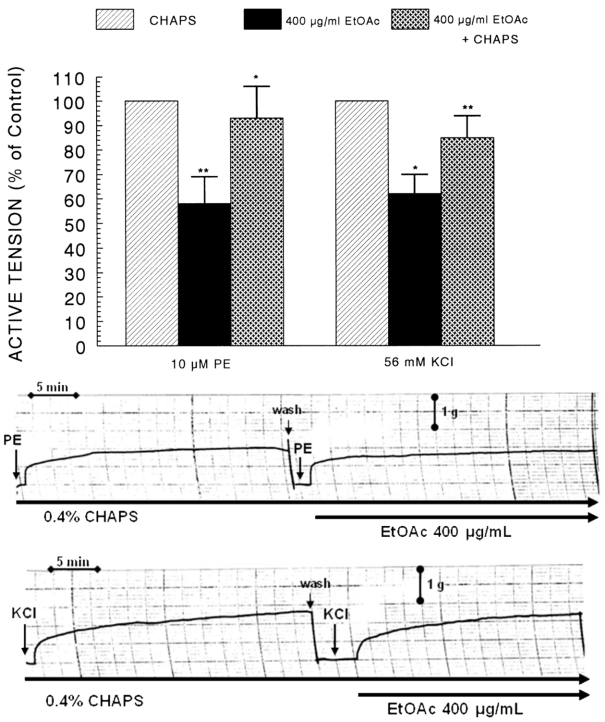

Fig. 4. Influence of CHAPS plus EtOAc fraction on contractile responses induced by phenylephrine (PE) and high potassium (KCl) (Upper) and the representative tracing of CHAPS plus EtOAc fraction effect on contractile responses induced by phenylephrine and high potassium in the isolated rat aortic strips (Lower). Other legends are the same as in Fig. 3. **: $P < 0.01$. At arrow marks, PE (10 μM) and KCl (56 mM) were added into a CHAPS-pretreated aortic strips. **Upper tracing**: PE-induced contractile response after EtOAc fraction (400 μg/ml)-treatment in a CHAPS-pretreated aortic strip. **Lower tracing**: High KCl-induced contractile response after EtOAc fraction (400 μg/ml)-treatment in a CHAPS-pretreated aortic strip. The chart speed was 5 mm/min.

inhibitor.[4] These results suggest that PCRC can inhibit the CA release at least partly through the activation of nNOS in the adrenal medulla of SHRs. Therefore, in the presence of L-NAME, it was likely interesting to compare the effects of EtOAc fraction on the contractile responses induced by high potassium and phenylephrine.

In the simultaneous presence of EtOAc fraction (400 μg/ml) and L-NAME (300 μM), the aortic contractile response evoked by phenylephrine (10^{-5} M) was recovered to 95 ± 11% ($P < 0.05$, $n = 7$) of the control in comparison with the inhibitory response of EtOAc fraction-treatment alone (58 ± 11%) from the resting tension level as shown in Fig. 3. High potassium (5.6×10^{-2} M)-induced contractile response in the simultaneous presence of EtOAc fraction (400 μg/ml) and L-NAME (300 μM) was recovered to 107 ± 4% ($P < 0.01$, $n = 8$) of the corresponding control compared with the inhibitory response of EtOAc fraction-treatment alone (62 ± 8%) from the resting tension level (Fig. 3).

Influence of the EtOAc fraction plus CHAPS on contractile responses induced by phenylephrine and high potassium in the thoracic aortic strips of normotensive rat – As shown in Fig. 3, EtOAc fraction-induced vasorelaxation was markedly blocked in the presence of L-NAME, a NO synthase inhibitor. Therefore, it is likely interesting to examine the effects of CHAPS, a detergent which suppresses endothelial function,[8] on EtOAc fraction-induced inhibitory responses to the contractile active tension evoked by high potassium and phenylephrine.

In the presence of EtOAc fraction (400 μg/ml) after pretreatment with 0.4% CHAPS, the aortic contractile response evoked by phenylephrine (10^{-5} M) exhibited 93

± 13% ($P < 0.01$, n = 5) of the control in comparison with the corresponding control response (100%) from the resting tension level as shown in Fig. 4. High potassium (5.6×10^{-2} M)-induced contractile response in the simultaneous presence of EtOAc fraction (400 μg/ml) after pretreatment with CHAPS elicited 85 ± 9% ($P < 0.01$, n = 4) of the control in comparison with the corresponding control response (100%) from the resting tension level (Fig. 4).

Effects of intravenous EtOAc fraction on blood pressure in the anesthetized normotensive rats – All of rats used in this study were allowed to be stabilized at least for 60 min before experimental protocols were initiated. When cardiovascular parameters were stabilized, EtOAc fraction (0.3 - 3.0 mgkg) was given into a femoral vein of the normotensive rat anesthetized with thiopental sodium and urethane. EtOAc fraction produced a dose-related and potent fall in arterial blood pressure. However, an equivalent volume of 0.9% saline given into a femoral vein did not produce any changes in blood pressure of the normotensive rats. As shown in Fig. 5, intravenous 0.3 mg of EtOAc fraction induced a fall in mean arterial pressure by 7.1 ± 1.6 mmHg from the original baseline of 121.4 ± 5.0 mmHg, but increasing doses of EtOAc fraction to 1.0 and 3.0 mg/kg, i.v. showed the decreased mean arterial pressure of 12.5 ± 1.8 and 26.6 ± 1.3 mmHg, respectively from the pre-injection level of the baseline from 10 rats. All of the above experimental results were statistically significant from the corresponding pre-injection values ($p < 0.01$).

Influence of phentolamine, chlorisondamine, L-NAME and sodium nitroprusside on the EtOAc fraction-induced depressor action – In 7 rats, in order to examine the relationship between adrenergic α-receptors and EtOAc fraction-induced depressor action, phentolamine (1.0 mg/kg) was given intravenously after obtaining the control responses of intravenous EtOAc fraction. In the presence of phentolamine effect, depressor response induced by intravenous EtOAc fraction (1.0 mg/kg) were greatly depressed to −3.0 ± 1.0 mmHg ($P < 0.05$) from the pre-injection level of the baseline as compared with the control depressor response (−19.1 ± 4.5 mmHg) as shown in Fig. 6 (A and B). Chlorisandamine (1.0 mg/kg), an autonomic ganglionic blocking agent was given slowly into a femoral vein. Following the administration of chlorisondamine, the baseline of blood pressure was reduced from 115.5 ± 5.2 mmHg to 69.5 ± 4.1 mmHg. In 6 rats, intravenous EtOAc fraction (1.0 mg/kg)-induced depressor response after chlorisondamine-treatment was markedly inhibited by 1.8 ± 0.5 mmHg ($P < 0.05$) as

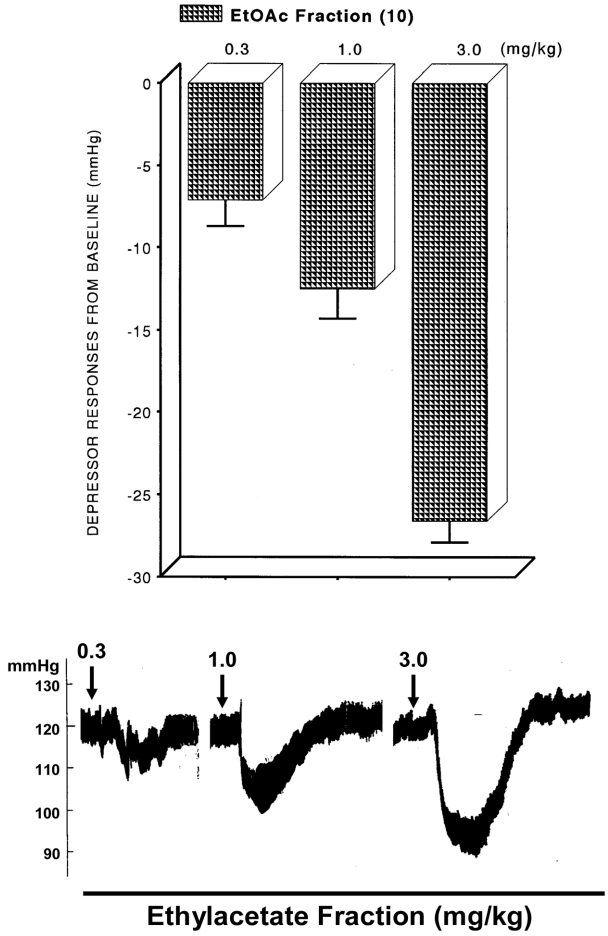

Fig. 5. Dose-dependent hypotensive effects of EtOAc fraction (Upper) and the typical tracings of EtOAc fraction-induced hypotensive action in an anesthetized rat (Lower). EtOAc fraction (0.3, 1.0 and 3.0 mg/kg, respectively) was administered into a femoral vein. Arterial blood pressure from pre-injection level was expressed in mmHg. EtOAc fraction at the indicated doses (0.3, 1.0 and 3.0 mg/kg) was injected intravenously at the arrow marks.

compared with the control depressor response (19.7 ± 4.9 mmHg), as shown in Fig. 6 (A and C). Intravenous infusion of L-NAME (3 mg/kg/30 min), an inhibitor of NO synthase, into a femoral vein resulted in a significant decrease in the blood pressure by 3.9 ± 0.7 mmHg ($P < 0.01$, n = 20) as compared with the control depressor response (15.5 ± 1.5 mmHg), as shown in Fig. 6 A and D). In 8 rats, in order to examine the relationship between NO and EtOAc fraction-induced depressor action, sodium nitroprusside (30 μg/kg/30 min) was infused intravenously after obtaining the control responses of intravenous EtOAc fraction. In the presence of sodium nitroprusside effect, depressor response induced by intravenous EtOAc fraction (1.0 mg/kg) were greatly depressed to 3.9 ± 0.8 mmHg ($P < 0.01$) as compared with the control depressor response (19.3 ± 2.7 mmHg) as shown in Fig. 6 (A and E).

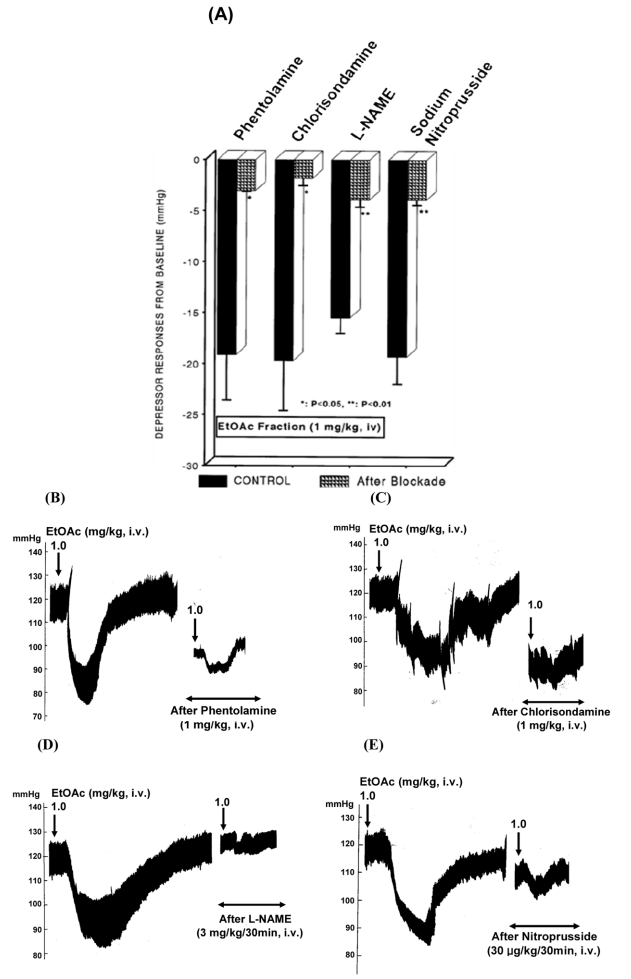

Fig. 6. Effects of phentolamine, chlorisondamine, L-NAME and nitroprusside on EtOAc fraction-induced hypotensive action in the anesthetized rats (A), and typical tracings showing the influence of phentolamine (B), chlorisondamine (C), L-NAME (D) and nitroprusside (E) on EtOAc fraction-induced hypotensive responses. Phentolamine (1 mg/kg), chlorisondamine (1 mg/kg), L-NAME (3 mg/kg/30 min) and sodium nitroprusside (30 μg/kg/30 min) were given intravenously, respectively, after obtaining EtOAc fraction-induced hypotensive action. Statistical difference was analyzed by comparing control response with that after treatment with each blockade. **: $P < 0.01$.

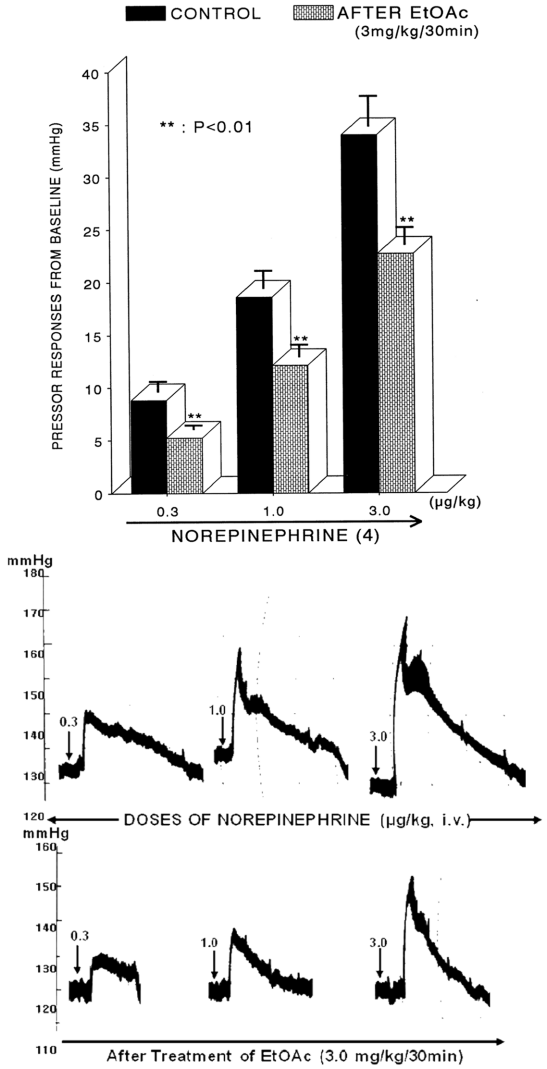

Fig. 7. Influence of intravenous EtOAc fraction on norepinephrine (NE)-evoked pressor responses (Upper) and the representative tracing of effect of EtOAc fraction on intravenous NE-induced pressor responses in anesthetized rats (Lower). EtOAc fraction (1.0, 3.0 and 10.0 mg/kg/30 min, respectively) was given intravenously after obtaining the corresponding control responses of intravenous NE (0.3, 1.0 and 3.0 μg/kg, respectively). **: $P < 0.01$. At arrow marks, the indicated doses (0.3, 1.0 and 3.0 μg/kg) of NE were administered into a femoral vein. Upper: NE-induced hypertensive responses in a non-treated rat. Lower: NE-induced hypertensive responses in a EtOAc fraction-pretreated rat. EtOAc fraction was infused into a femoral vein with a rate of 3 mg/kg/30 min. Arterial blood pressure from pre-injection level was expressed in mmHg. The chart speed was 10 mm/min.

Influence of intravenous EtOAc fraction on norepinephrine (NE)-evoked pressor responses in the anesthetized rats – As shown in Fig. 2 and 6 (A and B), EtOAc fraction greatly inhibited phenylephrine-induced contractile response of the aortic strip of normotensive rats, and also EtOAc fraction-induced depressor responses were significantly reduced by phentolamine and chlorisondamine, it suggests that EtOAc fraction might cause hypotension through the blockade of peripheral adrenergic á-receptors. It is also of interest to examine the effect of EtOAc fraction on norepinephrine-evoked pressor responses. When cardiovascular parameters were stabilized for 30 min before the experimental protocols were initiated, the administration of physiological saline solution in a volume of 0.2 ml into a femoral vein did not cause any changes in arterial blood pressure. Then, it was tried to test the effect of EtOAc fraction on norepenephrine-induced hypertensive responses in the anesthetized rats.

In 11 rats, as shown in Fig. 7, norepinephrine at doses of 0,3, 1.0 and 3.0 μg/kg, i.v. caused dose-dependent pressor responses of 8.8 ± 1.0 mmHg, 18.6 ± 1.7 mmHg and 34.0 ± 2.92 mmHg from the original baseline (121.4 ± 4.2 mmHg), respectively. After infusion of EtOAc fraction with a rate of 3.0 mg/kg/30 min, hypertensive responses of norepinephrine at doses of 0,3, 1.0 and 3.0 μg/kg were inhibited maximally to 59 ± 4% ($P < 0.01$), 65 ± 6% ($P < 0.01$) and 68 ± 5% ($P < 0.01$) of control responses at the above same doses, respectively.

Discussion

The present experimental results demonstrate that EtOAc fraction causes vasorelaxation in the isolated aortic strips of SHRs as well as normotensive rats at least partly by the increased NO production through the activation of NO synthase of vascular endothelium, but not through the activation of cyclooxygenase. In support of this idea, recently, it has been demonstrated that EtOAc fraction inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats[3] and spontaneously hypertensive rats.[4] It seems that this inhibitory effect of EtOAc fraction is exerted by inhibiting both the Ca^{2+} influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store partly through the increased NO production due to the activation of nitric oxide synthase.[3,4] In the present study, EtOAc fraction elicited a concentration-dependent inhibition in phenylehrine-induced contractile responses of rat aortic rings with functional endothelium. This effect was greatly abolished in the absence of functional endothelium by treatment with CHAPS, which is a detergent for removal of endothelium, indicating that the vasodilator effect of EtOAc fraction is dependent on endothelium-derived relaxing factors. To evaluate the participation of NO in the vasorelaxant activity of EtOAc fraction, aortic rings were treated with L-NAME, a classical NO synthase inhibitor. In the present experimental condition, the EtOAc fraction-induced vasodilatation was markedly blocked, as similarly observed in endothelium-denuded aortic rings by CHAPS, suggesting that NO is the main endothelium-derived relaxing factor involved in EtOAc fraction activity. The present results are fully in accordance with previous those findings obtained from red wines and grapes. Previously, it has been reported that red wines and grapes exhibit endothelium-dependent relaxation of blood vessels via enhanced generation and/or increased biological activity of NO, leading to the elevation of cGMP levels.[12,13] *In vivo* the polyphenol compounds of red wine (PCRW) were shown to reduce blood pressure in normotensive and hypertensive rats.[14-16] In denuded aortic rings, PCRW concentration 103-fold higher was necessary to induce relaxation.[17,18] The mechanisms underlining NO-dependent vasorelaxation caused by PCRW were investigated.[6,13,19] In addition to the increased NO synthase activity, PCRW may prolong the half-life and increase the bioavailability of NO, by reducing its degradation mediated by reactive oxygen species.[20] It has also been that Provinol elicited endothelium-dependent relaxation of rat femoral artery by the Ca^{2+}-induced increase of NO synthase activity and by protecting NO from degradation.[13] Yu and his colleagues[4] have found that PCRW inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats. It seems that this inhibitory effect of PCRW is mediated by blocking the influx of both ions through Na^{+} and Ca^{2+} channels into the rat adrenomedullary chromaffin cells as well as by inhibiting the release of Ca^{2+} from the cytoplasmic calcium store, which are due at least partly to the increased NO production through the activation of nitric oxide synthase.

PCRW and a grape skin extract also reduced blood pressure in males in several models of experimental hypertension,[16,21-24] which was related to a combination of vasodilator and antioxidant actions. Pechanova and his colleagues[21] also provided evidence that Provinols partially prevents L-NAME-induced hypertension, cardiovascular remodeling and vascular dysfunction via the increase of NO-synthase activity and prevention of oxidative stress. In the present study, intravenous EtOAc fraction-induced hypotensive response was significantly inhibited by pretreatment with L-NAME or sodium nitroprusside. In light of these results, it seems that EtOAc fraction may produce hypotensive action at least through the increased NO production by eNOS activation. Thus, in view of the beneficial effects of plant polyphenols, the present results of EtOAc fraction should shed light on the fact that the unique components of EtOAc fraction may contribte to the treatment or prevention of hypertension through their complex influence on the NO balance in the cardiovascular system.

Generally, it is well known that potassium chloride (KCl) opens voltage-dependent calcium channels by depolarizing the cell membrane of vascular smooth

muscle, resulting in increased influx of extracellualr Ca^{2+}.[25,26] Kim and his colleagues[27] have shown that the contractile responses of vascular smooth muscle induced by $CaCl_2$ and KCI may result most likely from the increased influx of extracellular Ca^{2+} through the voltage-dependent calcium channels (VDCCs). VDCCs are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. In the present work, incubation with EtOAc fraction inhibited KCl concentration-dependent contractile response in rat aortic strips. This result is consistent with the effect of 17-â estradiol on a large elastic aorta as in previous report[28,29] and is also supported by another study.[30] These findings suggest that EtOAc fraction may have Ca^{2+} antagonistic properties and can inhibit extracellular Ca^{2+} influx through VDCCs, which are similar to those of 17-β estradiol or resveratrol. Generally, the mechanism of potassium-induced vasoconstriction has been shown to be through the calcium-influx by the opening of the voltage-dependent calcium channels.[31] Voltage-dependent calcium channel blockers such as nifedipine or verapamil have been reported to attenuate potassium-induced vasoconstriction.[32] The contractile activity of vascular smooth muscle cells is mainly regulated by control over the cytoplasmic calcium concentration and both intracellular and extracellular calcium pools.[32] Based on these findings, the present results that EtOAc fraction inhibited high K^+-evoked contractile responses, and that the inhibitory effect of EtOAc fraction on high K^+-evoked contractile responses was enhanced, although their data are not shown here, indicate that EtOAc fraction may block the VDCCs in aortic smooth muscle cells.

In the present study, the findings that EtOAc fraction-induced hypotension is suppressed by the pretreatment with an autonomic ganglionic blocker (chlorisondamine), and adrenergic α-blocker (phentolamine) suggest strongly that the EtOAc fraction-induced hypotension may be mediated through the inhibition of sympathetic tone. The action site of EtOAc fraction seems to be the sympathetic ganglia or more higher level because its hypotensive response is inhibited by prior treatment of chlorisondamine. Furthermore, in terms of the fact that intravenous EtOAc fraction-evoked hypotension is significantly attenuated by adrenergic α-receptor blockade (phentolamine), and that EtOAc fraction inhibits greatly the pressor responses of norepinephrine, it is considered that EtOAc fraction causes the hypotensive action via the blockade of adrenergic α_1-receptors. Among drugs which interfere with peripheral sympathetic function, adrenergic α-receptor blocking agents alone cause reversal of the epinephrine pressor response.[33] When epinephrine is administered to untreated animals, its α-agonist properties predominate, resulting in a rise in mean arterial pressure. However, in the presence of adrenergic α-receptor blockade, the peripheral β_2-agonist properties of epinephrine predominate and a fall in arterial pressure or reversal of the pressor response is observed. In contrast, the pressor responses to norepinephrine are impaired by adrenergic α-receptor blockade, but are not reversed[34] as this agent processes little β_2-agonist activity.[35] These previous facts support that EtOAc fraction-induced depressor action may be due to the blockade of adrenergic α-receptors in the periphery. In the present work, EtOAc fraction also inhibited the norepinephrine-induced pressor responses as well as phenylephrine-evoked contractile responses in aortic strips isolated from SHRs and normotensive rats. These results suggest that EtOAc fraction may elicit the antagonistic activity of adrenergic α_1-receptors.

Based on all these results, many studies strongly support the view that polyphenol-rich diet, such as *Rubus coreanum* and red wine, could improve endothelial function and that the mechanisms of this beneficial effect found in above discussed *in vitro* studies (especially increased NO) might be involved *in vivo*, both in patients and in animals.

In conclusion, the present study provides conclusive data showing for the first time that EtOAc fraction elicits the endothelium- and NO-dependent vasorelaxation, which are due to unique polyphenolic constituents of EtOAc fraction that may augment eNOS activity and thus facilitates endothelial NO output, and suggesting that EtOAc fraction might be helpful in treating or alleviating cardiovascular diseases, such as hypertension and angina pectoris. The identification of the responsible constituents should help in the design of strategies to prevent or to improve cardiovascular diseases. In addition, in terms of these data, it is expected that intake of sports beverage containing active components extracted from *Rubus coreanum* is helpful to physiologically stabilize cardio-vascular system of athletes as well as beneficial to enhance athlete performance.

Acknowledgements

This study was supported partly by a research grant from Chosun University, Gwangju, Korea (2010-2011).

References

(1) Lee, J. W.; Do, J. H. *Korean J. Food & Nutr.* **2000**, *13,* 453-459.

(2) Cho, Y. J. *Korean J. Food & Nutr.* **2005**, *34,* 790-796.
(3) Kee, Y. W.; Lim, D. Y. *Arch. Pharm. Res.* **2007**, *30,* 1240-1251.
(4) Yu, B. S.; Ko, W. S.; Lim, D. Y. *Biomolecules & Therapeutics* **2009**, *16,* 147-160.
(5) Fitzpatrick, D. F.; Hirschfield, S. L.; Coffey, R. G. *Am. J. Physiol.* **1993**, *265,* H77-78.
(6) Andriambeloson, E.; Stoclet, J. C.; Andriantsitohaina, R. *J. Cardiovasc. Pharmacol.* **1999**, *33,* 248-254.
(7) Bouloumié, A.; Bauersachs, J.; Linz, W. *Hypertension* **1997**, *30,* 934-941.
(8) Moore, P.; Kal-Swayeh, O. A.; Chong, N. W.; Evans, R. A.; Gibson, A. *Br. J. Pharmacol.* **1990**, *99,* 408-412.
(9) Tallarida, R. J.; Murray, R. B. Manual of pharmacologic calculation with computer programs. 2nd Ed; Speringer-Verlag; New York, 1987.
(10) Lim, H. J.; Min, S. Y.; Woo, E. R.; Lim, D. Y. *Biomolecules &Therapeutics* **2011**, *19,* 477-486.
(11) Wada, A.; Takara, H.; Izumi, F.; Kobayashi, H.; Yanagihara, N. *Neuroscience* **1985**, *15*, 283-292.
(12) Fitzpatrick, D. F.; Fleming, R. C.; Bing, B.; Maggi D. A.; O'Malley, R. *J. Agric. Food Chem.* **2000**, *48,* 6384-6390.
(13) Zenebe, W.; Pecháòová, O.; Andriantsitohaina, R. *Physiol. Res.* **2003**, *52,* 425-432.
(14) Mizutani, K.; Ikeda, K.; Kawai Y.; Yamori, Y. *J. Nutr. Sci. Vitaminol (Tokyo).* **1999**, *45,* 95-106.
(15) Diebolt, M.; Bucher, B.; Andriantsitohaina, R. *Hypertension* **2001**, *38,* 159-165.
(16) Bernátová, I.; Pecháòová, O.; Babál, P.; Kyselá, S.; Stvrtina, S.; Andriantsitohaina, R. *Am. J. Physiol* (*Heart Circ Physiol).* **2002**, *282,* H942-H948.
(17) Ndiaye, M.; Chataigneau, T.; Andriantsitohaina, J. C. S.; Schini-Kerth, V. B. *Biochem. Biophys. Res. Commun.* **2003**, *310,* 371-377.
(18) Corder, R.; Douthwaite, J. A.; Lees, D. M.; Khan, N. Q.; Viseu Dos Santos, A. C.; Wood, E. G.; Carrier, M. J. *Nature* **2001**, *414,* 863-864.
(19) Martin, S.; Andriambeloson, E.; Takeda, K.; Andriantsitohaina, R. *Br. J. Pharmacol.* **2002**, *135,* 1579-1587.
(20) de Aetano, G.; Cerletti, C. *Nutr. Metab. Cardiovasc. Dis.* **2001**, *11,* 47-50.
(21) Pechanova, O.; Bernatova, I.; Babal, P.; Martinez, M. C.; Kysela, S.; Stvrtina, S.; Andriantsitohaina, R. *J. Hypertens.* **2004**, *22*, 1551-1559.
(22) Sarr, M.; Chataigneau, M.; Martins, S.; Schott, C.; El Bedoui, J.; Oak, M. H.; Muller, B.; Chataigneau, T.; Schini-Kerth, V. B. *Cardiovasc. Res.* **2006**, *71*, 794-802.
(23) Soares de Moura, R.; Costa Viana, F. S.; Souza, M. A. V.; Kovary, K.; Guedes, D. C.; Oliviera, E. P.; Rubenich, L. M.; Carvalho, L. C.; Oliveira, R. M.; Tano, T.; Gusmao Correia, M. L. *J. Pharm. Pharmacol.* **2002**, *54*, 1515-1520.
(24) Jiménez, R.; López-Sepúlveda, R.; Kadmiri, M.; Romero, M.; Vera, R.; Sánchez, M.; Vargas, F.; O'Valle, F.; Zarzuelo, A.; Dueñas, M.; Santos-Buelga, C.; Duarte, J. *Free Radic. Biol. Med.* **2007**, *43,* 462-473.
(25) Dube, G. P.; Baik, Y. H.; Schwartz, A. *J. Cardiovasc. Pharmacol.* **1985**, *7,* 377-389.
(26) Dube, G. P.; Baik, Y. H.; Van Breemen, C.; Schwartz, A. *Eur. J. Pharmacol.* **1988**, *145,* 39-47.
(27) Kim, J. M.; Park, K. O.; Baik, Y. H. *Chonnam. J. Med. Sci.* **1989**, *2,* 50-59.
(28) Li, H. F.; Li, W.; Zheng, T. Z.; Qu, S. Y.; Zhang, C. L. *Arch. Gynecol. Obstet.* **2002**, *266,* 101-104.
(29) Li, H. F.; Tian, Z. F.; Qiu, X. Q.; Wu, J. X.; Zhang, P.; Jia, Z. J. *Physiol. Res*. **2006**, *55,* 365-372.
(30) Nevala, R.; Korpela, R.; Vapaatalo, H. *Life Sci.* **1998**, *63*, 95-100.
(31) Spedding, M.; Paoletti, R. *Pharmacol. Rev.* **1992**, *44*, 363-376.
(32) Triggle, D. J.; Zheng, W.; Hawthorn. M. et al. *Ann. NY. Acad. Sci*. **1989**, *560*, 215-229.
(33) Constantine, J. W.; Mcshane, W. K.; Scriabine, A.; Hess, H. J. In Analysis of the hypotensive action of prazosin, Hypertension: Mechanisms and Management; Onesti, G.; Kim, K. E.; Moyer, J. H. Ed; Grume & Stratton Inc; New York, 1973.
(34) Freis, E. D.; Mackay, J. C.; Oliver, W. F. *Circulation* **1951**, *3,* 254-259.
(35) Ablad, B.; Borg, K. O.; Carlsson, E.; Johnson, G.; Malmfors, L.; Regardh, C. G. *Acta. Pharmacol. Toxicol (Copenh).* **1975**, *36,* 7-23.

Received May 20, 2014
Revised July 6, 2014
Accepted July 16, 2014