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Pine Needle Extracts Inhibit Contractile Responses of the Isolated Rat Aortic Strips[#]

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Abstract – The aim of the present study was to investigate whether self-fermented pine extract for 2 years (SFPE2) and ethyl acetate (EtOAc) fraction from self-fermented pine needle extract may affect the contractility of the isolated aortic strips and blood pressure of normotensive rats. SFPE2 (360-1440 μg/mL) significantly depressed both phenylephrine (10 μM)- and high potassium (56 mM)-induced contractile responses of the isolated rat aortic strips in dose-dependent fashion. The EtOAc-fraction (400 μg/mL) also inhibited both phenylephrine (10 μM)- and high potassium (56 mM)-induced contractile responses. Also, in anesthetized normotensive rats, intravenous injection of the EtOAc fraction (1.0~10.0 mg/kg) dose-dependently elicited hypotensive responses. The EtOAc fractions (1.0 and 3.0 mg/kg/30 min) inhibited norepinehrine-induced pressor responses. Intravenous infusion of SFPE2 fraction (3.0 and 10.0 mg/kg/30 min) also inhibited norepinehrine-induced pressor responses in both anesthetized spontaneously hypertensive rats (SHRs) and normotensive rats. In conclusion, these results suggest that both SFPE2 and the EtOAc fraction cause vascular relaxation in the aortic strips isolated from normotensive rats and SHRs as well as vasodepressor responses. Based on these experimental data, it seems that SFPE2 or the EtOAc fraction possesses active antihypertensive components, which are available to prevent or treat hypertension in future.
Keywords – Pine needle extract, Vasodepressor response, Antihypertensive component

Introduction

Red pine, the *Pinus densiflora* Sieb. et Zucc. (Pinaceae) grows naturally or is planted in mountain regions of Korea, Japan and China. Red pine needles have traditionally been used as a nourishing tonic drug in Korean folk medicines and are frequently used to brew a tea in Korea. It has been reported that pine needle extract shows several actions, such as an antioxidant activity in rats fed highly oxidized fat (Lee, 2003), cytotoxic effects on several cancer cell lines (Chung *et al*., 2002), inhibition of the pacemaker currents of interstitial cells of Cajal (ICC) by activating ATP-sensitive K^+ channels via the production of PGs (Cheong *et al*., 2005), and nitrite scavenging activities (Park *et al*., 2002). Hsu and his co-workers (2005) noted that pine (*Pinus morrisonicola* Hay.) needle scavenges superoxide and inhibits the growth of leukemia cell U937. It has been shown that the antioxidant activity potential of the several fractions from *Pinus densiflora* was in the order of ethyl acetate > *n*-butanol > water > dichloromethane fraction (Jung *et al*., 2003). Fitzpatrick and his co-workers (1998) have found that the pine bark extract was able to stimulate in vitro the production of nitric oxide, thus counteracting the vasoconstriction by adrenaline or noradrenaline in isolated aortic rings from rats. Pine pollen powder, called 'natural micro-nutrient storeroom', is rich in many kinds of body-demanding amino acid, minerals, vitamin, enzyme, and flavonoids (Wang *et al*., 2005). Pollen lipids of a pine species have been shown to cause a remarkable inhibition of platelet activating factor activity (Siafaka-Kapadai *et al*., 1986). Lee *et al*. (2009) suggested that pine pollen is a potential antioxidant and beneficial for inflammatory conditions through down-regulation of IL-1β-induced JNK and matrix metalloproteinases (MMPs). As a kind of Chinese traditional medicine, pine pollen, which is the male spore of pine tree, has been used as a drug and food for thousands of years. Different from bee pollen, pine pollen is collected artificially, and it has the characteristics of a single pollen source, pure quality and stable component.

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Apart from this, no reports regarding the cardiovascular effects of self-fermented pine extract (SFPE2) and ethylacetate fraction (EtOAc) derived from pine tree needle have been found so far. The present study therefore was designed to investigate the effects of SFPE2 and EtOAc fraction extracted from pine tree needles on blood pressure of the anesthetized rat and contractile responses of the isolated rat aortic strips, and to establish their mechanism of actions.

Materials and methods

Experimental procedure – Mature male Sprague-Dawley rats or spontaneously hypertensive rats, weighing 200 to 350 grams, were used in the experiment. The animals were housed individually in separate cages, and food (Cheil 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 aortic strips – The thorax was opened by a midline incision, and placing three hook retractors exposed the heart and surrounding area. 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 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.

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

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°C. The composition (mM) of Krebs was: NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MaCl_2$, 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 experiments were started. Vasoconstrictors were administered into the bath in order to obtain dose-response curves. In the subsequent experiments, under the presence of pine needle extract, some vasoconstrictors were administered, respectively. The data were expressed

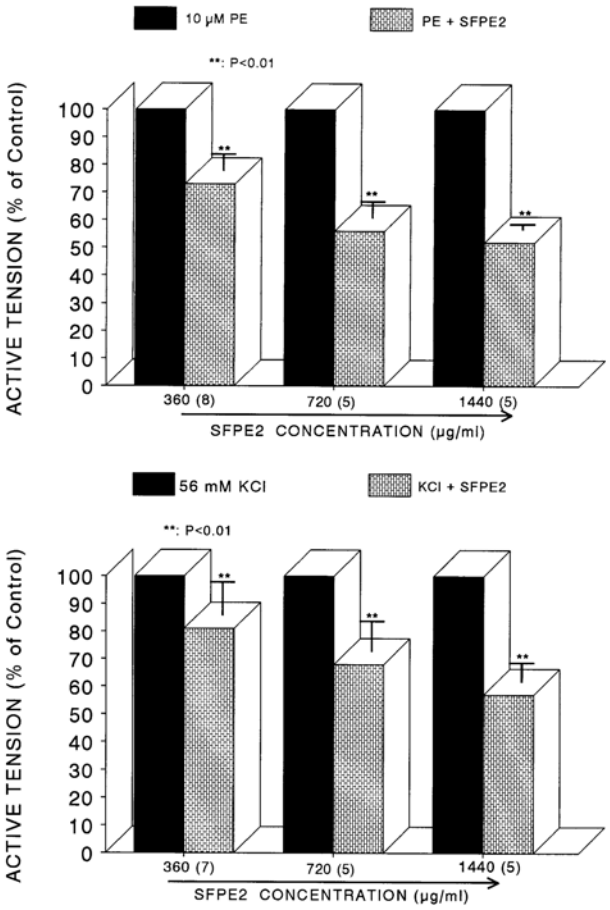

Fig. 1. Upper: Influence of self-fermented pine needle extract for 2 years (SFPE2) on phenylephrine (PE)-induced contractile responses in the isolated rat aortic strips. The contractile response was induced by adding 10 μM PE after adaptation with normal Krebs solution for two hours prior to initiation of the experimental protocol. "Black column" and "brick column" denote active tension induced evoked by PE before (control) and after adding 360, 720, and 1440 μg/ml of SFPE2, respectively. Numeral in the parenthesis indicates number of experimental rat aortic strips. Vertical bars represent the standard error of the mean (S.E.M). Ordinate: the active tension (% of control). Abscissa: concentrations of SFPE2 (μg/ml). Statistical difference was obtained by comparing the control with the SFPE2-pretreated group. **: $P < 0.01$. **Lower**: Influence of SFPE2 on high potassium-induced contractile responses in the isolated rat aortic strips. High potassium (56 mM) was added into the bath before and after pretreatment with 360, 720, and 1440 μg/ml of SFPE2, respectively.

as % of the control tension.

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 significance between 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 (1987).

Preparation of pine needle fractions – The leaves of Korean red pine trees (*Pinus densiflora* Sieb. et Zuc.) cultivated in Gokseong, Jeollanam-Do Province, Korea and harvested in 1999~2009 were collected to prepare for extraction. Pine needles were washed 3-4 times with tap water; dipped with charcoal water, dried, and ground for 1 minute to homogenized with con was allowed to put for 3 hours at 4 °C and the supernatant was recovered. This supernatant sample was stored at 4 °C for assay. Fresh pine needle extract (PE) was stored for years that favored emergence of microorganisms, which finally enabled spontaneous fermentation in extracts. The effects of the extract were examined for PE as well as after 2 years of self-fermentation designing as self-fermentation pine needle extract 2 years old (SFPE 2). The extract was freezing-dried to obtain solid sample. Finally the combined extract was freeze-dried. This dried sample is referred to as the pine needle extract.

Drugs and their sources – The following drugs were used: SFPE2 and EtOAc (maunufactured in our lab), phenylephrine hydrochloride, potassium chloride (KCl), and norepinephrine bitartrate (Sigma Chemical Co., U. S. A.), thiopental sodium and heparin sodium (Daehan Choongwae Pharm. Co., Korea). Drugs were dissolved in distilled water (stock) and added to the normal Krebs or saline solution as required. Concentrations of all drugs used are expressed in terms of molar base and g.

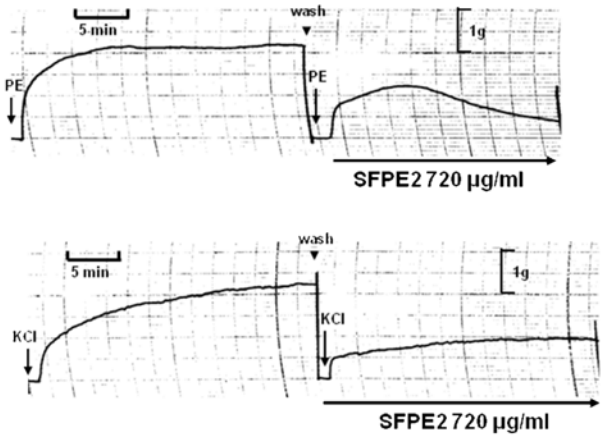

Fig. 2. Upper: The typical tracing showing the effect of SFPE2 on phenylephrine (PE)-induced contractile responses in the rat aortic strips. Left: PE-induced contractile response. Right: PE-induced contractile response in the presence of 720 µg/ml of SFPE2. At arrow mark, the indicated dose (10^{-5} M) of phenylephrine was added to the bath. **Lower**: The typical tracing showing the effect of SFPE2 on high potassium (KCl)-induced contractile responses in the rat aortic strips. Left: KCl-induced contractile response. Right: KCl-induced contractile response in the presence of 720 µg/ml of SFPE2. At arrow mark, the indicated dose (56 mM) of KCl was added to the bath. The chart speed was 5 mm/min.

Results

Effects of SFPE2 on contractile responses induced by phenylephrine (PE) and high K^+ in the isolated rat aortic strips – The resting tension from the isolated rat aortic strips 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 SFPE2 on PE- as well as high K^+ chloride-mediated contractile responses in the rat aorta were examined. In the present study, SFPE2 itself did not produce any effect on the resting tension in the aortic strips isolated from the rat (data not shown). However, it was found that there is difference in the contractile responses induced by PE and high K^+ after pretreatment with SFPE2 as shown in Fig. 1 and 2.

When 10^{-5} M of PE concentration was administered into the aortic bath, its active tension amounted to 2.1 ± 0.2 g from the resting tension level. However, under the pre-existence of SFPE2 at 360, 720 and 1440 µg/ml,

respectively, SFPE2 dose-dependently inhibited 10^{-5} M-PE-induced tension to 73 ± 6% ($P < 0.01$), 56 ± 6% ($P < 0.01$) and 52 ± 2% ($P < 0.01$) of the corresponding control responses, respectively (Fig. 1-upper and 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 (Wada *et al.*, 1985). When added through the bath, high potassium at the concentration of 5.6×10^{-2} M, which is a membrane-depolarizing concentration, caused an increase in aortic contraction. As shown in Fig. 1 (lower) and 2, high potassium (5.6×10^{-2} M)-induced contractile responses after pre-loading with 360, 720 and 1440 μg/ml of SFPE2, respectively, were also significantly attenuated to 81 ± 12% ($P < 0.01$), 68 ± 11% ($P < 0.01$) and 57 ± 7% ($P < 0.01$) of the control responses, respectively, compared with the corresponding control response (1.6 ± 0.2 g) in dose-dependent fashion.

Effects of EtOAc fraction on contractile responses induced by PE and high K^+ in the isolated rat aortic strips – Since SFPE2 was found to inhibit the contractile response of PE and high potassium in the isolated rat aortic strips, it was likely interesting to compare the effects of EtOAc fraction on the contractile responses induced by high potassium and PE. In the presence of 400 μg/ml of EtOAc fraction, the aortic contractile response evoked by PE (10^{-5} M) was 42% of the control in comparison with the corresponding control response (2.2 ± 0.3 g) from the resting tension level as depicted in Fig. 3. High potassium-induced contractile response before treatment with EtOAc fraction was 2.0 ± 0.1 g, while after pretreatment with 400 μg/ml of EtOAc fraction it was markedly reduced to 40% of the corresponding control response, which there was statistically difference between control and EtOAc fraction-treated groups ($P < 0.01$).

Effects of intravenous EtOAc fraction on arterial blood pressure in the anesthetized rats – 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, EtOAc fraction injected intravenously to the normotensive thiopental-anesthetized rat produced a dose-dependent decrease in arterial blood.

In 6 rats, as shown in Fig. 4, intravenous 1.0 mg/kg of EtOAc fraction produced a fall in arterial blood pressure to –4.8 ± 0.3 mmHg ($P < 0.01$) from the original baseline of 122 ± 6 mmHg. Increasing intravenous doses of EtOAc fraction to 3.0 and 10.0 mg/kg caused the dose-dependent

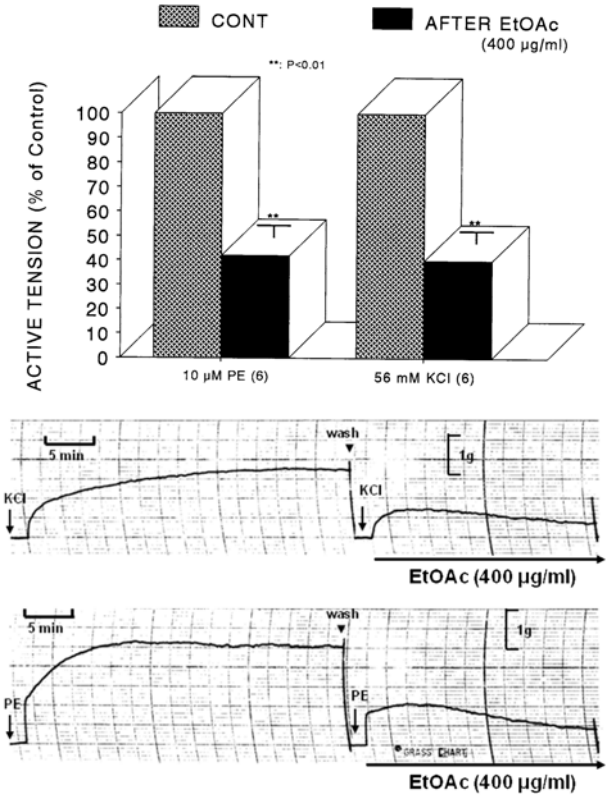

Fig. 3. Upper: The typical tracing showing the effect of EtOAc fraction on high KCl- (upper panel) and phenylephrine (PE, lower panel)-induced contractile responses in the rat aortic strips. Left: High KCl- or PE-induced contractile response. Right: High KCl- or PE-induced contractile response in the presence of 400 μg/ml of EtOAc fraction. At arrow mark, the indicated dose of High KCl- or PE was added to the bath. The chart speed was 5 mm/min. **Lower:** Influence of ethyl acetate fraction (EtOAc) on phenylephrine (PE)- and high potassium-induced contractile responses in the isolated rat aortic strips. The contractile response was induced by adding 10 μM PE (left) or 56 mM KCl (right) before (carpet column) and after adding 400 μg/ml of EtOAc fraction. Other legends are the same as in Fig. 1 and 2. **: $P < 0.01$.

reduction in arterial pressure responses to –14.8 ± 0.9 mmHg ($P < 0.01$) and –24.7 ± 1.3 mmHg ($P < 0.01$) from the original baseline, respectively.

Effects of EtOAc fraction on norepinephrine-induced pressor responses in normotensive rats – Since EtOAc fraction greatly inhibited PE-induced contractile responses of the isolated aortic smooth muscle as shown in Fig. 3, it was of interest to examine the effect of intravenous 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. In 8 rats, norepinephrine at doses of 0, 3, 1.0 and 3.0 μg/kg caused dose-dependent pressor responses of 14 ± 1.2 mmHg, 27.1 ±

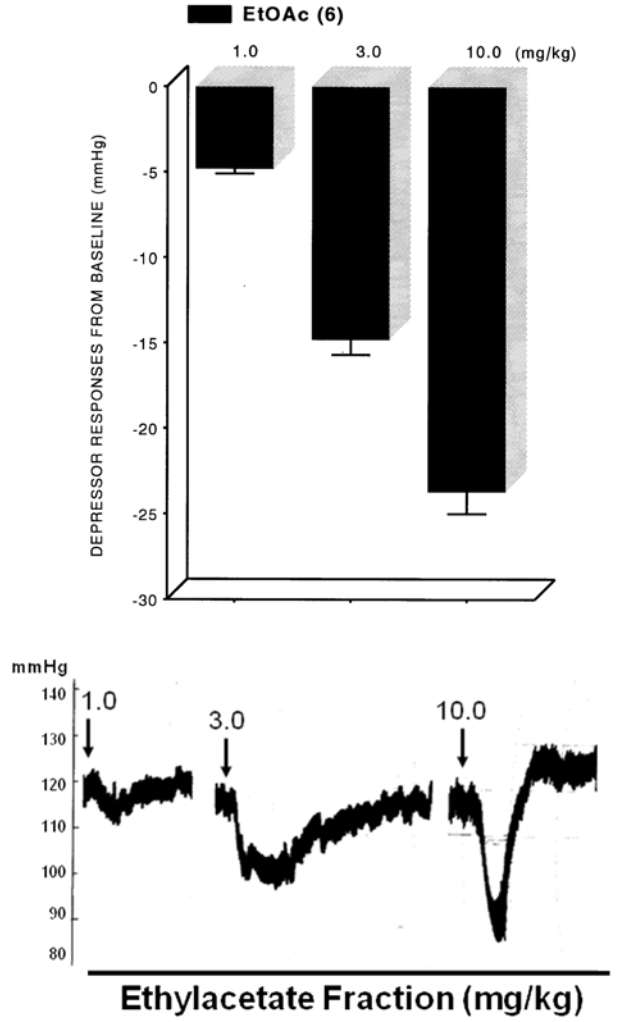

Fig. 4. Upper: Influence of intravenous EtOAc fraction on arterial blood pressure in the thiopental-anesthetized rats. Ordinate: changes of arterial blood pressure in mmHg from 6 rats. Abscissa: intravenous doses of EtOAc fraction in mg/kg. Vertical bar on each column indicates standard error of mean (S.E.M.). There was statistically significant difference in changes of arterial pressure responses induced by EtOAc fraction from pre-injection level. Numeral in the parenthesis denotes Number of animals used in the experiment. **Lower:** The representative tracing of the intravenous EtOAc fraction-induced depressor response in the thiopental-anesthetized rat. At arrow mark, the indicated doses (1, 3 and 10 mg/kg) of EtOAc fraction were administered into a femoral vein at 20 - 30 min interval. The chart speed was 20 mm/min. The original base-line of arterial blood pressure was 122 ± 6 mmHg.

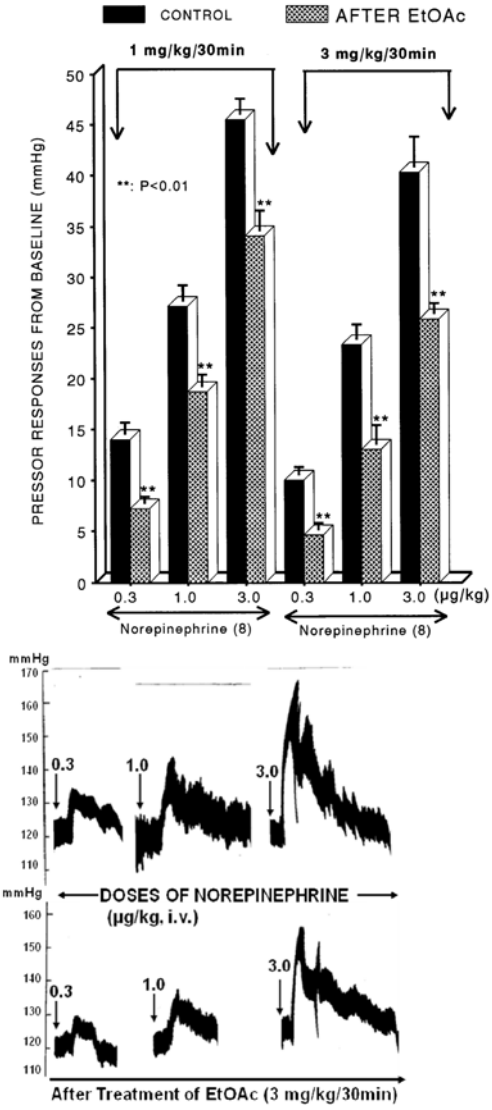

Fig. 5. Upper: Influence of EtOAc fraction on intravenous norepinephrine (NE)-induced pressor response in anesthetized normotensive rats. Ordinate: Changes of blood pressure from baseline level in mmHg. Abscissa: Intravenous doses of NE in μg/kg. Vertical bar on top of each column indicates standard error of mean (S.E.M.). Statistical difference was obtained by comparing the control with the EtOAc fraction-pretreated group. The original base-line of arterial blood pressure was 122 ± 13 mmHg. **: P < 0.01. **Lower**: The representative tracing of EtOAc fraction's effect on intravenous NE-induced pressor response in the anesthetized normotensive rat. At arrow marks, the indicated doses (1.0, 3.0 and 10.0 μg/kg) of NE were administered into a femoral vein. Upper panel: NE only-induced hypertensive responses in a non-treated rat. Lower panel: NE-induced hypertensive responses in the EtOAc fraction-pretreated rat. EtOAc fraction was infused into a femoral vein with a rate of 1 and 3 mg/kg/30 min. The chart speed was 20 mm/min.

1.6 mmHg and 45.5 ± 1.8 mmHg from the original baseline (122 ± 13 mmHg), respectively. However, after infusion of EtOAc fraction with a rate of 1.0 mg/kg/30 min, they were significantly depressed to 51 ± 5% (P < 0.01), 69 ± 4% (P < 0.01) and 75 ± 4% (P < 0.01) of the corresponding control responses at the above same doses, respectively (Fig. 5-upper). Also, after the pretreatment of EtOAc fraction with a rate of 3.0 mg/kg/30min in 8 rats they were greatly inhibited to 46 ± 7% (P < 0.01), 56 ± 8% (P < 0.01) and 64 ± 3% (P < 0.01) of the corresponding

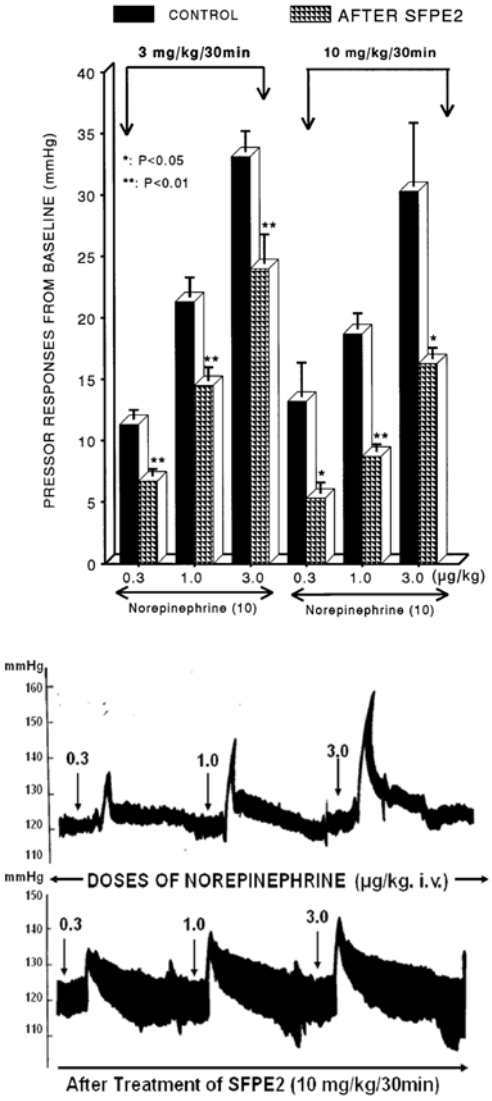

Fig. 6. Upper: Influence of SFPE2 on intravenous norepinephrine (NE)-induced pressor response in anesthetized normotensive rats. The original base-line of arterial blood pressure was 124 ± 11 mmHg. *: $P < 0.05$, **: $P < 0.01$. **Lower**: The representative tracing of SFPE2's effect on intravenous NE-induced pressor response in the anesthetized normotensive rat. Other legends are the same as in Fig. 5. SFPE2 was infused into a femoral vein with a rate of 3 and 10 mg/kg/30min. The chart speed was 20 mm/min.

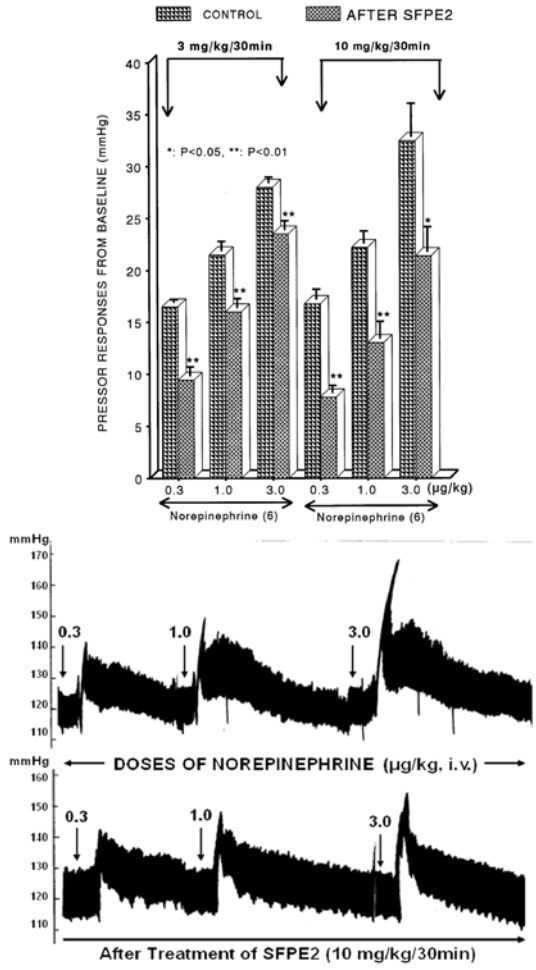

Fig. 7. Upper: Influence of SFPE2 on intravenous norepinephrine (NE)-induced pressor response in spontaneously hypertensive rats (SHRs). The original base-line of arterial blood pressure was 188 ± 12 mmHg. *: $P < 0.05$, **: $P < 0.01$. **Lower**: The representative tracing of SFPE2's effect on intravenous NE-induced pressor response in the anesthetized SHR. Other legends are the same as in Fig. 5. SFPE2 was infused into a femoral vein with a rate of 3 and 10 mg/kg/30 min. The chart speed was 20 mm/min.

control responses (10.0 ± 0.8 mmHg, 23.3 ± 1.5 mmHg and 40.3 ± 3.0 mmHg) at the above same doses, respectively (Fig. 5-upper). Fig. 5 (lower) shows that norepinephrine-evoked pressor responses are greatly attenuated after pretreatment with intravenous EtOAc fraction (3.0 mg/kg/30 min).

Effects of SFPE2 on norepinephrine-induced pressor responses in normotensive rats and SHRs – In order to compare to effects of SFPE2 in normotensive rats and SHRs, we attempted to examine the effect of SFPE2 on norepinephrine-evoked pressor responses. In 10 normotensive rats, norepinephrine at doses of 0.3, 1.0 and 3.0 μg/kg before the pretreatment with SFPE2 caused dose-dependent pressor responses of 11.3 ± 0.8 mmHg, 21.3 ± 1.6 mmHg and 33.1 ± 1.7 mmHg from the original baseline (124 ± 11 mmHg), respectively. However, after infusion of SFPE2 with a rate of 3 mg/kg/30 min, they were significantly inhibited to 59 ± 5% ($P < 0.01$), 68 ± 5% ($P < 0.01$) and 73 ± 7% ($P < 0.01$) of the corresponding control responses at the above same doses, respectively (Fig. 6). Also, following the pretreatment of SFPE2 with a rate of 10.0 mg/kg/30 min in 10 rats, they were reduced to 40 ± 7% ($P < 0.05$), 47 ± 3% ($P < 0.01$) and 54 ± 3% ($P < 0.05$) of the corresponding control responses (13.2 ± 2.9 mmHg, 18.73 ± 1.3 mmHg and 30.3 ± 5.2 mmHg) at the above same doses, respectively (Fig. 6).

In 6 SHRs, norepinephrine at doses of 0.3, 1.0 and 3.0 μg/kg before the pretreatment with SFPE2 caused dose-dependent pressor responses of 16.5 ± 0.3 mmHg, 21.5 ± 0.9 mmHg and 28.0 ± 0.6 mmHg from the original baseline (188 ± 12 mmHg), respectively. However, after infusion of SFPE2 with a rate of 3 mg/kg/30min, they were significantly reduced to 57 ± 5% ($P < 0.01$), 74 ± 4% ($P < 0.01$) and 84 ± 3% ($P < 0.01$) of the corresponding control responses at the above same doses, respectively (Fig. 7-upper). Also, following the pretreatment of SFPE2 with a rate of 10.0 mg/kg/30min in 6 SHRs, they were reduced to 46 ± 7% ($P < 0.01$), 59 ± 8% ($P < 0.01$) and 66 ± 7% ($P < 0.05$) of the corresponding control responses (16.8 ± 1.0 mmHg, 22.2 ± 1.2 mmHg and 32.5 ± 3.2 mmHg) at the above same doses, respectively (Fig. 7-upper). Fig. 7 (lower) also shows that norepinephrine-evoked pressor responses in SHRs are markedly reduced following after pretreatment with intravenous infusion of SFPE2 (10.0 mg/kg/30 min).

Discussion

The present experimental results have demonstrated that intravenous infusion of SFPE2 causes a dose-dependent inhibitory action on norepinephrine-induced pressor responses in normotensive rats and SHRs, and that EtOAc fraction also produces depressor responses in normotensive rats as well as inhibitory action on norepinephrine-induced pressor responses in a dose-dependent fashion. Both EtOAc fraction and SFPE2 caused vascular relaxation in the thoracic aortic strips isolated from normotensive rats. Based on these data, it seems that both EtOAc fraction and SFPE2 may have vascular relaxation at least partly through the blockade of adrenergic α_1-receptors, in addition to the unknown mechanism of the direct vasorelaxation.

In support of these data, from methanol extract of the needles of *Pinus densiflora*, (+)-catechin was isolated as one of active principles, together with the inactive components, dihydrokaempferol, and 1-*O*-benzoyl glucoside (Choi *et al.*, 2001). Pine pollen powder, called 'natural micro-nutrient storeroom', is rich in many kinds of body-demanding amino acid, minerals, vitamin, enzyme, and flavonoids (Wang *et al.*, 2005). Pycnogenol (a standardized extract from the bark of the French maritime pine) consists of a concentrate of polyphenols. Main constituents are procyanidins, pharmacologically active biopolymers composed from units of catechin and epicatechin. Additionally, Pycnogenol contains the bioflavonoids catechin and taxifolin and a number of phenolic acids (Rohdewald, 2002). In *in vivo* studies, polyphenolic compounds isolated from red wine (PCRW) were shown to reduce blood pressure in normotensive and hypertensive rats (Mizutani *et al.*, 1999; Diebolt *et al.*, 2001). Extracts of tea (Fitzpatrick *et al.*, 1995) and flavonoids found in tea (Fitzpatrick *et al.*, 1993) have been shown to give vasodilator effects *in vitro*. In terms of these findings, the results obtained from the present study seem likely that both EtOAc fraction and SFPE2 containing polyphenolic compounds can cause the depressor effect. Hosseini and his co-workers (2001) have shown that In a double blind, placebo controlled, cross-over study with hypertensive patients, oral administration of Pycnogenol reduced systolic blood pressure from mildly hypertensive (140-159 mmHg) to normal values, but the reduction of diastolic blood pressure did not reach statistical significance Thus, both EtOAc fraction and SFPE2 seem to possess vasorelaxant effects, which may contribute to reduce blood pressure in hypertensive patients.

In general, among drugs which interfere with peripheral sympathetic function, adrenergic α-receptor blocking agents alone cause reversal of the epinephrine pressor response (Constantine *et al.*, 1973). 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 (Freis *et al.*, 1975) as this agent processes little β_2-agonist activity (Ablad *et al.*, 1975). In terms of the fact that PE-evoked contractile response is greatly depressed by EtOAc fraction or SFPE2, it is thought that both EtOAc fraction and SFPE2 have vascular dilatatory activity through the adrenergic α-receptor blockade. In view of these reports, in the present work, the finding that attenuation of norepinephrine-induced pressor responses in normotensive rats by both EtOAc fraction and SFPE2 as also in SHRs by EtOAc fraction demonstrates that both EtOAc fraction and SFPE2 may possess the antagonistic activity of adrenergic α_1-receptors. However, it has been suggested that antioxidant (Rice-Evans *et al.*, 1995) and vasodilatory (Fitzpatrick *et al.*, 1993; Fitzpatrick *et al.*, 1995) polyphenolics in tea can attenuate the transient pressor effect of caffeine, and lower blood pressure during regular consumption. Pycnogenol® has been used successfully for reduction of pathologically enhanced capillary walls permeability. In hypertensive rats administration of

Pycnogenol® dose-dependently strengthened weak capillary walls (Gábor *et al*., 1993). Fitzpatrick and his co-workers (1998) have reported that the pine bark extract was able to stimulate in vitro the production of nitric oxide, thus counteracting the vasoconstriction by adrenaline or noradrenaline in isolated aortic rings from rats. In a placebo-controlled, double-blind, parallel group study performed with 58 patients to investigate effects of Pycnogenol on patients with hypertension, supplementation of the patients with 100 mg Pycnogenol over a period of 12 weeks helped to reduce the dose of the calcium antagonist nifedipine in a statistically significant manner (Liu *et al*., 2004).

Several previous studies have shown that pine needle extract shows several actions, such as an antioxidant activity in rats fed highly oxidized fat (Lee, 2003), cytotoxic effects on several cancer cell lines (Chung *et al*., 2002), inhibition of the pacemaker currents of interstitial cells of Cajal (ICC) by activating ATP-sensitive K^+ channels via the production of PGs (Cheong *et al*., 2005), and nitrite scavenging activities (Park *et al*., 2002). Moreover, Pycnogenol, an extract formula from the bark of French maritime pine is found to exert significant antioxidant activity, and primarily comprises phenolic compounds (catechin, epicatechin, and taxifolin) and flavonoids (procyanidins) (Rohdewald, 2005). It has been reported that Pycnogenol causes a number of beneficial the cardiovascular effects. Previously, several studies have shown that Pycnogenol supplementation is associated with inhibition of platelet aggregation (Araghi-Niknam *et al*., 2000), decrease of low-density lipoprotein cholesterol (LDL-C) and increase of high-density lipoprotein cholesterol (Devaraj *et al*., 2002), and improvement of hypertension (Hosseini *et al*., 2001). Therefore, we hopefully expect that the both SFPE2 and EtOAc fraction, like Pycnogenol, a novel mixture of flavonoids, may reduce the antihypertensive medicine use and cardiovascular risk factors in hypertensive patients.

Generally, it is well known that high K^+ opens voltage-dependent calcium channels by depolarizing the cell membrane of vascular smooth muscle, resulting in increased influx of extracellualr Ca^{2+} (Bolton, 1979; Schwartz & Taira, 1983; Dube *et al*., 1985; 1988). Kim and his colleagues (1989) have shown that the contractile responses of vascular smooth muscle induced by $CaCl_2$ and high K^+ may result most likely from increased influx of extracellular Ca^{2+} through the voltage-dependent calcium channels. In terms of these results, the present findings that EtOAc fraction and SFPE2 inhibited the contraction of rat aortic smooth muscle evoked by PE (an α_1-adrenergic receptor agonist) as well as by high K^+ (a membrane depolarizer) suggest that vascular relaxation of EtOAc fraction and SFPE2 is mediated by the blockade of α_1-adrenergic receptors.

In previous studies, three cellular mechanisms have been proposed to explain relaxant response of vascular smooth muscle: (i) blockade of extracellular Ca^{2+} entry into cells (Fleckstein, 1977; Schwartz & Triggle, 1984), (ii) increase in binding or sequestration of intracellular Ca^{2+} (Watkins & Davidson, 1980; Imai & Kitagawa, 1981), and (iii) inhibiting the release of intracellular stored Ca^{2+} (Imai & Kitagawa, 1981; Ito *et al*., 1980a, b). In contrast, the contractions of vascular smooth muscles induced by neurohumoral agents have been found to be composed of two components: Phasic contraction induced by the Ca^{2+} released from inside the cell and tonic tension related to the Ca^{2+} influx (Bevan, 1982; Dube *et al*., 1988), both leading to increased intracellular calcium.

In the light of these facts, it could not be ruled out that EtOAc fraction and SFPE2 can relax the contractile responses of vascular smooth muscle evoked by PE through the blockade of extracellular Ca^{2+} entry into the muscle cells. Thus, these effects of EtOAc fraction and SFPE2 seem to contribute at least partly to the facts that pine needle extract reduces blood pressure in mild hypertensive patients (Hosseini *et al*., 2001). Extracts of tea (Fitzpatrick *et al*., 1995) and flavonoids found in tea (Fitzpatrick *et al*., 1993) have also been found to give vasodilator effects *in vitro*, and higher consumption of black tea was associated with lower systolic blood pressure (Stensvold *et al*., 1992). Moreover, it has been shown that (−) epicatechin also concentration-dependently relaxed U46619-contracted arteries without the functional endothelium. It is unlikely that (−) epicatechin acts as an antagonist at prostaglandin receptors to cause relaxation since it reduced arterial contraction induced by other vasoconstrictors, such as PE and endothelin 1 (Huang *et al*., 1998). The endothelium-independent relaxation induced by (−) epicatechin may be partly mediated through inhibition of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in vascular smooth muscle cells because (−) epicatechin significantly reduced the high K^+-induced contraction in the same preparation (Huang *et al*., 1998). Recently, it has been also found that (−) epicatechin could act on endothelium to increase intracellular Ca^{2+} and nitric oxide release, which may account for the endothelium-dependent relaxation (Huang *et al*., 1999).

Based on concentrations used in this study, the pharmacological activities of EtOAc fraction is more potent than those of SFPE2. In support of this finding, it

has been shown that the antioxidant activity potential of the several fractions from *Pinus densiflora* Sieb. et Zucc. (Pinaceae) was in the order of ethyl acetate > *n*-butanol > water > dichloromethane fraction (Jung *et al*., 2003). The ethyl acetate soluble fraction exhibiting strong antioxidant activity was further purified by repeated silica gel and Sephadex LH-20 column chromatographies. An active lignan (+)-isolarisiresinol xylopyranoside, as well as two active flavonoids kaempferol 3-*O*-β-galactopyranoside and its 6-acetyl derivative], were isolated.

Taken together, these experimental results demonstrate that intravenous infusion of SFPE2 causes a dose-dependent inhibitory action on norepinephrine-induced pressor responses in normotensive rats and SHRs, and that EtOAc fraction also produces depressor responses in normotensive rats as well as inhibitory action on norepinephrine-induced pressor responses in a dose-dependent fashion. Both EtOAc fraction and SFPE2 caused vascular relaxation in the thoracic aortic strips isolated from normotensive rats. It seems that both EtOAc fraction and SFPE2 may have vascular relaxation at least partly through the blockade of adrenergic α_1-receptors, in addition to the unknown mechanism of the direct vasorelaxation. Based on the active profile exposed through several experiments, it can be concluded that both EtOAc fraction and SFPE2 show strong vasorelaxant and depressor activity. These two pine needle extracts, therefore, may be useful in developing new herbal medicine against cardiovascular diseases and might be relevant for clinical use for hypertension.

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