2010년 2월
석사학위 논문

Characterization and purification of fibrinolytic compounds during fermentation of pine needle extract

조선대학교 대학원

생 명 공 학 과

정 주 영

Characterization and purification of fibrinolytic compounds during fermentation of pine needle extract

솔잎 발효에 따른 혈전분해 물질 정제 및 생리적 특성

2010년 2월 25일

조선대학교 대학원

생 명 공 학 과

정 주 영

Characterization and purification of fibrinolytic compounds during fermentation of pine needle extract

지도교수 정 현 숙

이 논문을 이학 석사학위신청 논문으로 제출함

2009년 10월

조선대학교 대학원

생 명 공 학 과

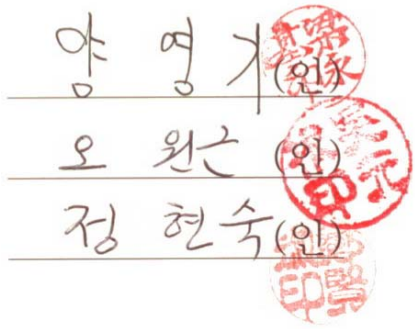
정 주 영

정주영의 석사학위논문을 인준함

위원장　　조선대학교 교수

위　원　　조선대학교 교수

위　원　　조선대학교 교수



2009년 11월

조선대학교 대학원

Characterization and purification of fibrinolytic compounds during fermentation of pine needle extract

A thesis submitted to the Graduate School
of the Chosun University in partial fulfillment of the requirement
for the degree of Master of Science

by

Juyoung Jung

Department of Biotechnology, Graduate School,
Chosun University, Gwangju, Korea

2009. 11. 26

Approved by:

Prof. Young-Ki Yang, Ph.D.

Prof. Won Keun Oh, Ph.D.

Major Advisor:

Prof. Hyeonsook Cheong, Ph.D.

CONTENTS

III. RESULTS

LIST OF SCHEMES

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACh	acetylcholine
BAY-K8644	6-dimethyl-3-nitro-4-(2-trifluoro- methyl-phenyl) -pyridine-5-carboxylate
BuOH	butanol
CA	catecholamine
EF-SFPE 4	ethyl acetate fractions isolated from SFPE 4
EtOAc	ethyl acetate
F-SFPE 4	fractions isolated from SFPE 4
NE	norepinephrine
MeOH	methanol
PE	fresh Pine needle Extract
SFPE	Self-Fermentation Pine needle Extract
SHRs	Spontaneously Hypertensive Rats

국문초록

솔잎 발효에 따른 혈전분해 물질 정제 및 생리적 특성

정주영

조선대학교 대학원

생명공학과

지도교수 : 정현숙

솔잎은 예로부터 약리적 효능이 있어 장수와 건강을 위해 식용되어 왔으며, 오늘날 부가가치와 응용가치가 매우 높은 천연물로 알려져 있다. 따라서 본 연구는 솔잎발효액으로부터 silica gel (입자크기, 63-200 ㎛) 와 RP-18 (입자크기, 40-63 ㎛) 및 preparative HPLC [ODS-H80 colum (20 × 150 ㎜, 입자크기 4 ㎛)] 등의 각종 크로마토그래피 방법을 사용하여 혈전분해 활성이 우수한 화합물을 분리하였고 분리된 화합물에 대하여 각종 스펙트럼 방법 (NMR, UV)과 보고된 문헌을 참고하여 구조를 규명하였다. 분리된 화합물 중 화합물 1과 2는 유리형 페놀산 계열의 화합물로서 protocatecuic acid (1)와 shikimic acid (2)임을 확인하였다. 또한, 솔잎발효액에서 추출한 분획의 유효성분 탐색을 위해서 분획을 이용하여 정상혈압 흰쥐의 적출부신 관류모델에서 카테콜아민 분비에 대한 영향, 적출대동맥편의 수축반응에 대한 영향, 전신혈압반응에 대한 영향을 검색하였다. 그 결과 솔잎발효액분획중 물분획과 부탄올분획은 정상혈압 흰쥐의 적출 관류부신에서카테콜아민분비에 아무런 작용을 미치지 않았으나 에틸아세테이트분획이 콜린성 수용체 흥

분작용 및 직접 막탈분극 작용에 의한 CA분비반응을 억제함을 증명하였다. 이러한 EtOAc분획의 억제작용은 SDs의 부신수질 크롬친화세포에서 전압의존성 L형 칼슘통로를 차단함으로써 칼슘유입을 봉쇄함으로써 나타나는 것으로 사료된다. 또한 솔잎발효액(SPFE 5) 및 EtOAc분획이 정상혈압 흰쥐의 적출 대동맥편에서 선택성 α_1-아드레날린 수용체 작동제인 페닐에프린과 고칼륨에 의한 수축반응에 대해 이완작용을 나타낸다는 사실을 증명하였다. 그리고 솔잎발효액(SPFE 5) 및 EtOAc분획이 정상혈압마취 흰쥐에서 뚜렷한 용량의존성 혈압하강 반응을 나타내었고 마취 한 자연발증고혈압 흰쥐에서 솔잎발효액(SPFE 5)을 대퇴정맥 내로 주입한 후에 교감신경말단으로 부터 유리되는 신경전달 물질로써 혈압상승을 일으키는 노르에피네프린(NE)의 승압반응이 뚜렷이 억제됨을 알 수 있었다. 이로써 본 연구의 결과는 솔잎발효액(SPFE 5)과 EtOAc분획이 혈압하강 작용이 있음을 시사한다. 따라서 솔잎 발효액으로부터 분리된 화합물들은 고혈압, 협심증 등을 비롯한 심혈관계 치료제로서 새로운 치료제 개발에 유익하게 이용 될 수 있다고 제안한다.

ABSTRACT

Characterization and purification of fibrinolytic compounds during fermentation of pine needle extract

Juyoung Jung

Advisor: Prof. Hyeonsook Cheong, Ph. D.

Department of Biotechnology,

Graduate School of Chosun University

The Korean pine needle has been used to beverage manufacture and medicine. During this screening effort we found that EtOAc-soluble and BuOH-soluble extract of SFPE activated fibrinolytic activity, two compounds from the of SFPE were isolated by bioassay-guided isolation using silica gel (63-200 ㎛ particle size), RP-18 (40-63 ㎛ particle size) column chromatograohy, and preparative HPLC [ODS-H80 colum (20 × 150 ㎜, 4 ㎛ particle size)]. Their structures were elucidated on the basic of spectral (including NMR, UV) and physicochemical analyses. Compounds 1 and 2 were free phenolic acid named as protocatecuic acid and shikimic acid. Also, in order to search mechanisms of action, influence of catecholamine release in the isolated rat adrenal gland, influence of contractile responses of

aortic strips isolated from rats, and influence of blood pressure of normotensive rat and SHRs will be used by abstracted fractions. In blood pressure lowering test using catacholamine (CA) secretion detection in white mouse, the CA secretion was inhibited by treatment EtOAc fraction however H_2O and BuOH fraction displayed no effect. In addition, the EtOAc fraction induced relaxation on aorta smooth muscle of the white mouse exposed to phenylephrine (PE) and reduced blood pressure raised by treatment of neurotransmitter norepinephrine (NE). These results suggest that there is possibility of getting up good materials from the SFPEs for beverage antihypertensive.

I. INTRODUCTION

Red pine, *Pinus densiflora Sieb. et Zucc.*, grows naturally or is planted in mountain regions of Korea, Japan, China, and the extreme southeast of Russia. It has been used for health and longevity. Today, it is famous for a natural substance useful and wonderful (Sung, 2004; Lee *et al.*, 2005). It has been presently used in treating the disease of adult people, diabetes, neuralgia, skin problems etc. in oriental medicine (Sung and Kim, 2005). Recently, in Korea, there has been growing public interest in incorporating pine needle-based products into the diet because of their health-promoting properties. It has been reported that pine needle extract improves unidentified clinical syndromes such as fatigue, depression, anxiety, sleeping disturbance, etc. (Ichikawa *et al.,* 1998; Wakanyaku Medical Institute, 1996). And, the pine needle extract inhibits the pacemaker currents of interstitial cells of Cajal by activating ATP-sensitive K^+ channels via the production of prostaglandins (Cheong *et al.,* 2005). In connection with this, the presence of epicatechin and p-coumaric acid in the EtOAc extract of pine needle may be partially responsible for the biological action of the EtOAc extract of pine needle. These results suggest that the ethyl acetate extract of pine needle may provide potential protective effects against LDL oxidation and attenuating excessive NO generation at inflammatory sites; consequently, this may contribute to anti-atherosclerotic and anti-inflammatory effects of the EtOAc extract of pine needle (Yen *et al.,* 2008).

Fibrin is the primary protein component of blood clots, which are formed from fibrinogen by thrombin (Voet and Voet, 1990). The insoluble fibrin fiber is

hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as tissue plasminogen activator (t-PA), a urokinase-type plasminogen activator, bloodl plasminogen activator, and streptokinase plasminogen complex (Collen, 1980; Collen and Lijnen, 1991). According to the report by the World Health Organization (WHO) in 2001, the proportion of deaths caused by cardiovascular diseases is 29%. In terms of embolism-related diseases like cerebral thrombosis and pulmonary thrombosis, fibrin is related to a high proportion of deaths. Because of its lethality, thrombolytic therapy has been extensively investigated as a medical remedy for these diseases. Despite their widespread use, all these agents have undesired side effects, exhibit low specificity for fibrin, and are also relatively expensive. Therefore, the searches for other fibrinolytic enzymes from various sources are being continued. Over the last decade, potent fibrinolytic enzymes have been discovered from a variety of sources, such as earthworms (Mihara *et al.,* 1991; Peng *et al.,* 2005), snake venoms (Jia *et al.,* 2003; De-Somone *et al.,* 2005), insects (Ahn *et al.*, 2003), mushroom (Lee *et al.*, 2006), and fermented food products like Japanese *natto* (Sumi *et al.,* 1992; Choi *et al.,* 2005), and Korean *chungkook-jang* soy sauce (Jeong *et al.,* 2001; Kim *et al.*, 2006). Because of the promising biological benefits for reducing blood clotting through the consumption of food sources containing fibrinolytic enzymes, many researchers have extensively explored new sources of fibrinolytic enzymes in the form of Asian fermented food products, with the purpose of providing potential health benefits for humans through the use of nutraceutical

ingredients (Hwang *et al.*, 2007). Nevertheless, very little information currently exists concerning the nutritional value, functional effects, and systematic evaluation of pine needle. In this study, the EtOAc-soluble and BuOH-soluble fraction of the self-fermentation pine needle extract (SFPE) were tested for active effects on fibrinolysis. And, I would like to describe the isolation and structure elucidation of these compounds and the evaluation of their fibrinolytic activity from SFPE. Also, we attempted to screen and identify the microorganism that has strong fibrinolytic activity from a fermented of pine needle extract.

On the one hand, the presence of polyphenolic compounds is widespread among plants and plant products (Formica and Regelson 1995; Zenebe and Pecháň iová2002). Several epidemiological studies have shown that consumption of foods rich in polyphenolic compounds is associated with lower incidence of cardiovascular disease. Despite these studies, there are no reports on *in vivo* functional effects of SFPE on the cardiovascular system. In order to search mechanisms of action, influence of catecholamin (CA) release in the isolated rat adrenal gland, influence of contractile responses of aortic strips isolated from rats, and Influence of blood pressure of normotensive rat and SHRs will be used by abstracted fractions.

II. MATERIALS AND METHODS

A-1. Preparation of PE and SFPEs

The leaves of Korean red pine trees (*Pinus densiflora* Sieb. et Zuc.) were cultivated in Gokseong, Jeollanam-Do Korea, and harvested in 1999~2009. Pine needles were cleaned 3, 4 times with tap water; dipped with charcoal water and dried and ground for 1 minute to homogenized with con was allowed to put for 3 hours at 4℃ and the supernatant was recovered. This supernatant sample was stored at 4℃ for assay. 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 fresh pine needle extract (PE) as well as after 4 and 10 years of self-fermentation designing as self-fermentation pine needle extract 4 years old (SFPE 4) and self-fermentation pine needle extract 10 years old (SFPE 10). The extract was freezing dried to obtain solid sample.

A-2. Chemicals and chromatography

Column chromatography was carried out using silica gel (63-200 ㎛ particle size) and RP-18 (40-63 ㎛ particle size) from MeOH. Precoated TLC silica gel 60 F_{254} plates from Merck were used for thin-layer chromatography. The HPLC were carried out using a Gilson system with a UV detector, and ODS-H80 column (20 × 150 ㎜, 4 ㎛ particle size, YMC Co., Lid., Japan).

NMR spectra were obtained on Varian Inova 500 MHz spectrometer with TMS as the internal standard. Deuterated solvent for NMR analysis $CDCl_3$ was purchased from CIL (Cambridge Icotope Lab., USA).

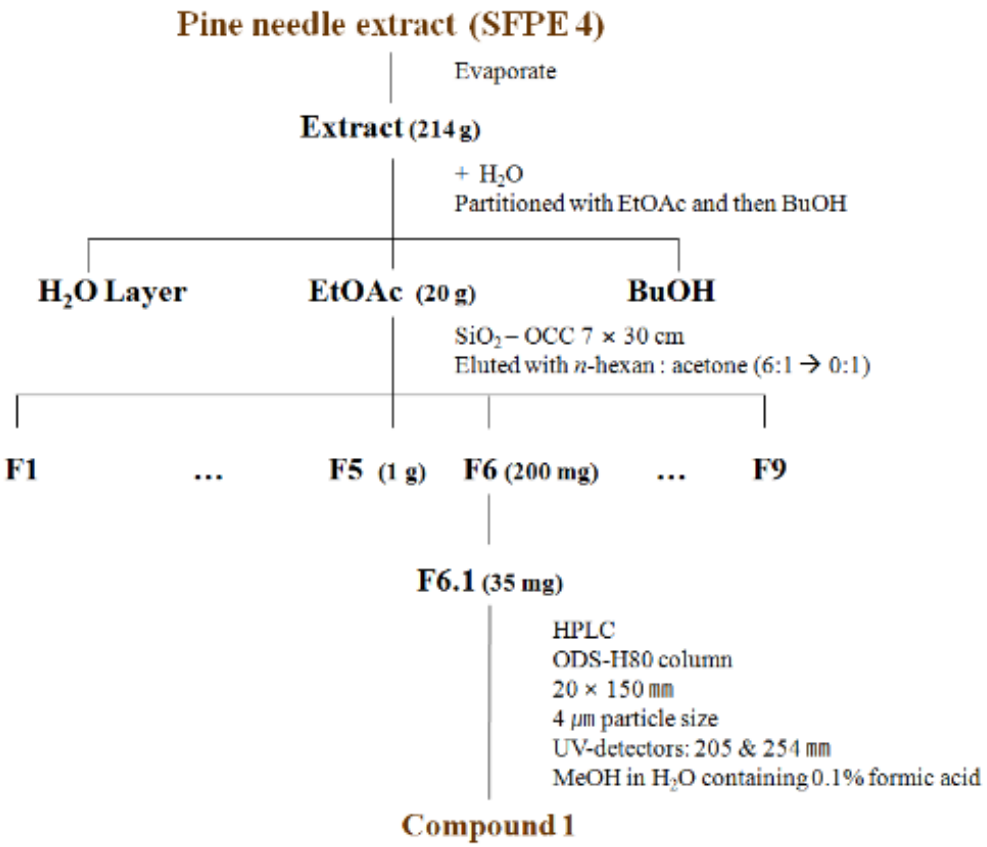
B. Purification of fibrinolytic active compound from SFPE

1. Isolation of fractions from SFPE

a. Isolation of fractions from SFPE 4

The soluble SFPE 4 were evaporated and concentrated to yield a dry residue (214 g). The extract was suspended in H_2O and partitioned with BuOH and EtOAc. The EtOAc-soluble fraction (20 g) was found to be most active among the solvent fraction. This fraction was fractionated by silica gel column chromatography (7 × 30 ㎝) using a gradient of *n*-hexane: acetone (from 6:1 to 0:1), to yield nine combined fractions according to their TLC profiles. The fibrinolytic activity was concentrated in F5 and F6 by *in vitro* assay. These active fractions were subjected to additional chromatography. Fraction F5 (I g) was continued applying to RP-18 silica gel column chromatography (7 × 25 ㎝), eluted using a gradient of MeOH/H_2O (1:2 to 1:0) to obtain five subfractions (F5.1-F5.5) based on TLC profile. Further isolation of combined fraction F.5.5 and F6 (200 ㎎) by HPLC on ODS-H80 column [(20 ×150 ㎜, 4 ㎛ particle size); solvent MeOH in H_2O containing 0.1% formic acid (0-25, 42-45 min: 33% MeOH, and 28-28 min: 100% MeOH); flow rate 3

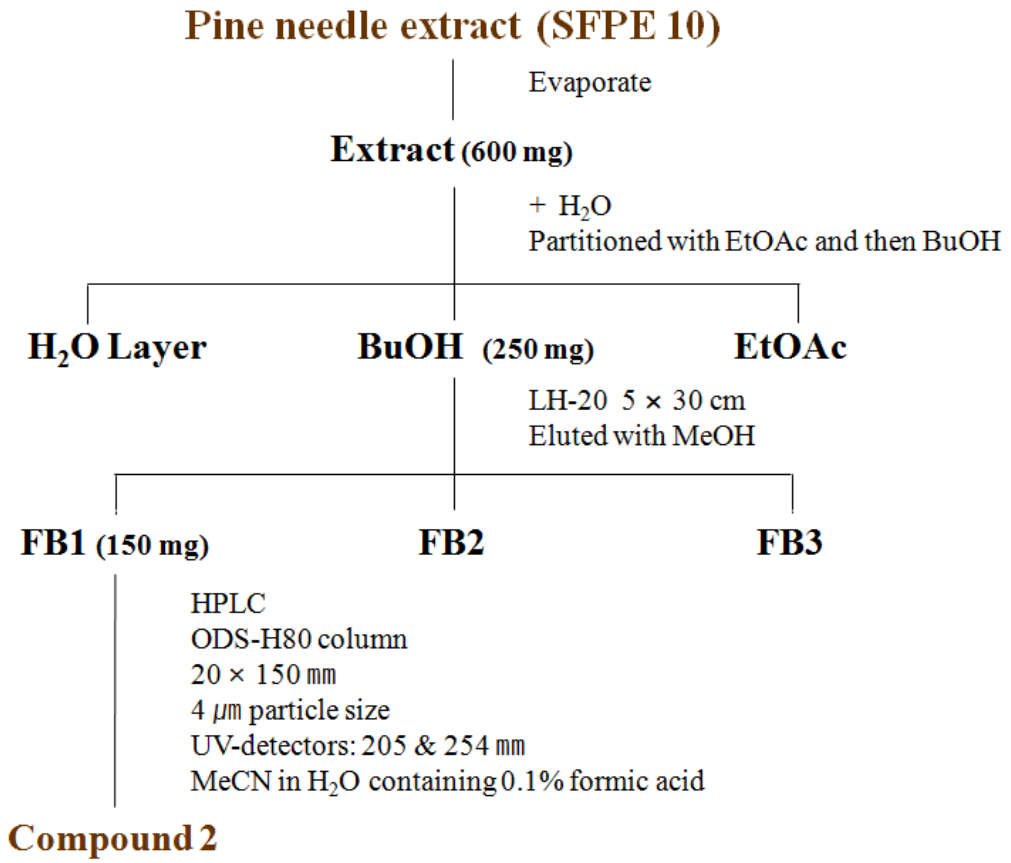
㎖/min; UV detection at 205 and 254 ㎚] resulted in the isolation of compound **1** (Scheme 1).



Scheme 1. Isolation of compound **1** from the EtOAc fraction of SFPE 4.

b. Isolation of compound 2 from SFPE 10

The soluble SFPE 10 were evaporated and concentrated to yield a dry residue (600 ㎎). The extract was suspended in H_2O and partitioned with BuOH and EtOAc. The fibrinolytic activity was concentrated in BuOH layer by *in vitro* assay. These active fractions were subjected to additional chromatography. The BuOH fraction (250 ㎎) was applied to Sephadex LH-20 column chromatography (5 × 30 ㎝) using MeOH as the eluting solvent to give three fractions (FB1 – FB3). FB1 fraction (150 ㎎) was separated by preparative HPLC on an ODS-H80 column [(20 × 150 ㎜, 4 ㎛ particle size); solvent MeCN in H_2O containing 0.1% formic acid (0-13, 21-25 min: 5% MeCN and 16-20 min: 80% MeCN); flow rate 3 ㎖/min; UV detection at 205 and 254 ㎚] resulted in the isolation of compound 2 (90 ㎎) (Scheme 2).



Scheme 2. Isolation of compound 2 from the BuOH fraction of SFPE 10.

c. The Fibrinolytic and fibrinogenolytic assays

Fibrinolytic activity was determined using the method described by Astrup and Mullertz (1952), with minor modifications. The fibrin agarose plate was made of a 1 ㎜ thickness and contained 15㎖ agarose solution (1% agarose in 20mM tris-HCl buffer, pH 8.0), 3 ㎖ of human fibrinogen solution, (30 ㎎/㎖ in 20mM tris-HCl buffer, pH 8.0) and 18 ㎕ thrombin solution (100 units/㎖, human thrombin in 20mM tris-HCl buffer, pH 8.0). The clot was allowed to stand for 1 hour at room temperature, and holes were made on the fibrin plate by using a capillary glass tube (5 ㎜ in diameter). Then 20 ㎕ of the sample solution was carefully placed onto the plate. The plate was incubated for 3~5 hours at 37°C and the diameter of the lytic circle was measured. An equal volume of streptokinase solution (100 units/㎖; Sigma) also was incubated as a control. Enzyme activity was estimated by measuring the width of the clear zone.

2. Identification of containing fibronolysis by microorganism from SFPE

a. Isolation of containing fibrinolysis microorganism

To study the dynamic pattern of microbial involvement during the fermentation of PE, the SFPEs were dissolved in 10 ㎖ of distilled water by each PE and SFPEs sample (100 ㎕). After serial dilution, samples were spread onto GPYA plates (glucose 40 g/L, peptone 5 g/L, yeast extract 5 g/L,

pH 5.0~5.2), and Nutrient plates (peptone 5 g/L, beef extract 3 g/L) followed by incubation at 28℃ and 37℃ for 48 hours. After incubation, highly mucoid and high growth colonies that appeared on the plates added 5% skim milk were incubated at 28℃ and 37℃ for 24 hours. Colonies showing the protease activity were isolated separately and cultured purely. The isolated strain was cultured in 5 ㎖ of liquid incubated at 28℃ and 37℃ for 24 hours of shaking at 130 rpm. Morphological and biological characteristic of a isolate were investigated according to fibrin plate method. FIbrin plate method was determined using the described by Astrup and Mullertz (1952).

b. PCR amplification of 16S rRNA and 26S rRNA

The amplification of the 16S rRNA gene and 26S rRNA gene each sample was performed using thermal cycler. The 16S rRNA gene and 26S rRNA was amplified by universal primer 16S BR1, BF2 and 26S YR1, YF2 Table 1. Each mixture (final volume, 20㎕) contained 1 ㎕ of template DNA, each primer at a concentration of 1 ㎕, each deoxynucleotide triphosphate (dNTP) at a concentration of 2 ㎕, 10 × Ex*Taq* buffer of 2 ㎕ and EX*Taq* polymerase of 0.2 ㎕ (Takara, Japan). Initial denaturation was carried out at 94℃ for 4 min, followed by 35 cycles consisting of denaturation at 94℃ for 30 sec, annealing at 57℃ for 30 sec, extension at 72℃ for 2 min and an additional final extension at 72℃ for 5 min. Based upon the result of the 16S rRNA sequence and 26S rRNA sequence of the isolate. A sequence similarity search was performed using BLAST in the NCBI database.

Primers	Sequence
BR1	5'-TACGGYTACCTTGTTACGAC-3'
BF2	5'-GAGTTTGATCCTGGCTCAG-3'
YR1	5'-GGTCCGTGTTTCAAGACGG-3'
YF2	5'-GCATATCAATAAGCGGAGGAAAAG-3'

Table 1. 16S rRNA and 26S rRNA primers used in this study.

C. Blood pressure lowering action in the compounds from SFPE 4

1. Influence of fractions isolated from SFPE 4 on catecholamine release in the isolated rat adrenal gland

a. Extraction of adrenal gland

The adrenal gland was isolated by some modification of the methods described previously (Wakade, 1981). Mature male Sprague-Dawley rats, weighing 200 to 250 grams, were used in the experiment. On the day of experiment, a rat was anesthetized with thiopental sodium (50 ㎎/㎏) intraperitoneally, and tied in supine position on fixing panel. The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by the placement of three-hook retractors. The stomach, intestine and portion of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauge pads an urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations. A cannula, used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of adrenal vein (if any), vena cava and aorta were ligated. Heparin-saline (400 IU/㎖) was injected into vena cava to prevent blood coagulation before ligating the vessels and cannulations. A small slit was made into the adrenal cortex just opposite the

entrance of the adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid escaped only from the slit made in adrenal cortex. Then the adrenal gland, along with ligated blood vessels and the cannula, was carefully removed from the animal and placed on a platform of a leucite chamber (Fig. 1).

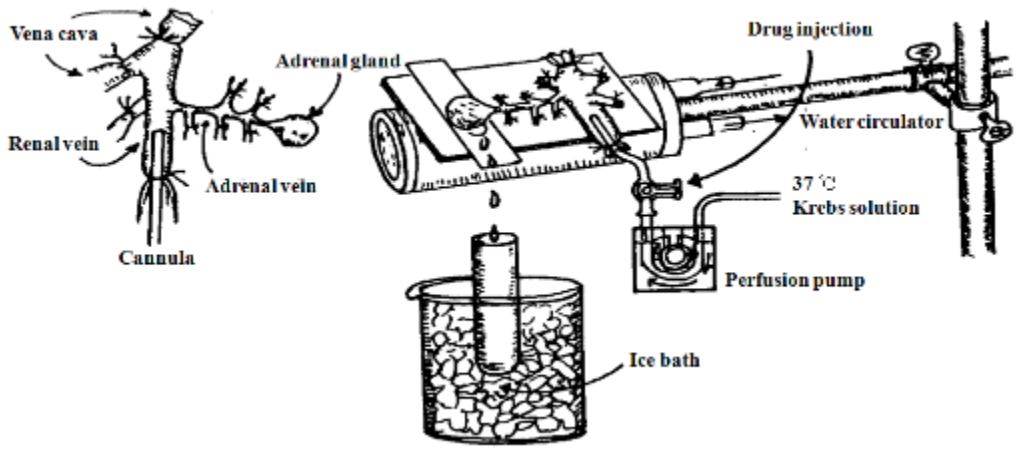


Fig 1. Schematic drawing if the preparation used to study secretion of catecholamines in the isolated perfused rat adrenal gland.

b. Perfusion of adrenal gland

The adrenal glands were perfused by means of peristaltic pump (ISCO® pump, WIZ Co., USA) at a rate of 0.31 ㎖/min. The perfusion was carried out with Krebs-bicarbonate solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.7. The solution was constantly bubbled with 95% O_2 + 5% CO_2 and the final pH of the solution was maintained at 7.4-7.5. The solution contained disodium EDTA (10 ㎍/㎖) and ascorbic acid (100 ㎍/㎖) to prevent oxidation of catecholamine.

c. Drug administration

The perfusion of Bay-K-8644 (10 μ M) for 4 min was made into perfusion stream, respectively. A single injection of ACh (5.32 mM) and KCl (56 mM) in a volume of 0.05 ㎖ was injected into perfusion stream via a three-way stopcock, respectively. In the preliminary experiments, it was found that, upon administration of the above drugs, secretory responses to ACh, KCl, and Bay-K-8644 returned to pre-injection level in about 4~8 min. Consequently, all the perfusates of afrenal was collected after medication during approximately 4~5 min. The made-up experiment has been finished, and adrenal medulla has confirmed function of the secretion. After that, following the experiment was performed through inserting ACh and Kreb solution. In order to reduce the partition of CA, we are collecting perfusate from adrenal

on ice bath.

d. Collection of perfusate

As a rule, prior to stimulation with various secretagogues, the perfusate was collected for 4 min to determine the spontaneous secretion of CA (background sample). Immediately after the collection of the background sample, collection of the perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated sample perfusate collected for 4 to 8 min. The amounts secreted in the background sample have been subtracted from that secreted from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures.

To study the effect of fractions isolated from SFPE 4 (F-SFPE 4) on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing F-SFPE 4 for 90 min, and then the perfusate was collected for a certain period (background sample). The medium was then changed to the one containing the stimulating agent then F-SFPE 4 was added, and the perfusate was collected for the same period as that for the background sample. The adrenal gland's perfusate was collected in chilled tubes.

e. Measurement of catecholamine

The content of the perfusate was measured directly by the fluorometric method of Anton and Sayre (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluorospectrophotometer (Kontron Co., Milano, Italy).

A volume of 0.2 ㎖ of the perfusate was used for the reaction. The CA content in the perfusate from the glands stimulated by secretagogues used in the present work was high enough to obtain readings several folds greater than the reading of the control samples (unstimulated). The sample blanks were also the lowest for perfusates of stimulated and non-stimulated samples. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

f. Drugs and their sources

The following drugs were used: F-SFPE4, acetylcholine chloride, potassium chloride, and methyl-1,4-dihydro-2,6-dimethyl- 3-nitro-4-(2-trifluoro-methyl-phenyl)-pyridine-5-carboxylate (BAY-K8644). Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except Bay-K-8644, which was 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 used, except F-SFPE4, are expressed in terms of molar base.

g. Statistical analysis

The statistical difference between the control and 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 (SEM). The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray (1987).

2. Influence of SFPE4 and EtOAc fractions isolated from SFPE 4 on contractile responses of aortic strips isolated from rats

a. Isolation of thoracic aortic strips

Mature male Sprague-Dawley rats, weighing 200 to 250 grams, were used in the experiment. On the day of experiment, a rat was anesthetized with thiopental sodium (50 ㎎/㎏) intraperitoneally, and tied in supine position on a fixing panel. 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 ㎜ length.

b. Experiments of vascular relaxation

The ring segment of aorta was mounted in a muscle bath by sliding the ring over two parallel stainless-steel hooks (0.15 ㎜ in diameter). The lower hook was fixed on bottom of the bath and the upper was connected to isometric transducer (Grass FT. 03). The ring was challenged with high potassium two times, and if it responded with contraction, the proper experiment was started. The signal from the transducer was displayed on a polygraph (Grass Instruments Model 79). Vasoconstrictors were administered into the bath in order to obtain dose-response curves. In the subsequent experiments, under the presence of SFPE 4 or EF-SFPE 4, some vasoconstrictors were administered, respectively.

c. Bath solution

We used Krebs-bicarbonate solution. 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. The volume of bath was 25 ㎖ and the bath solution was saturated with 95% O_2 and 5% CO_2 at 37℃ (Fig. 2).

d. Drug administration

During equilibration period of 2 hours, the resting tension was adjusted to 0.5 g. Bath liquid was exchanged every 30 min, and drug was given to inside Bath at intervals of 120 min. The data were expressed as % of the control tension.

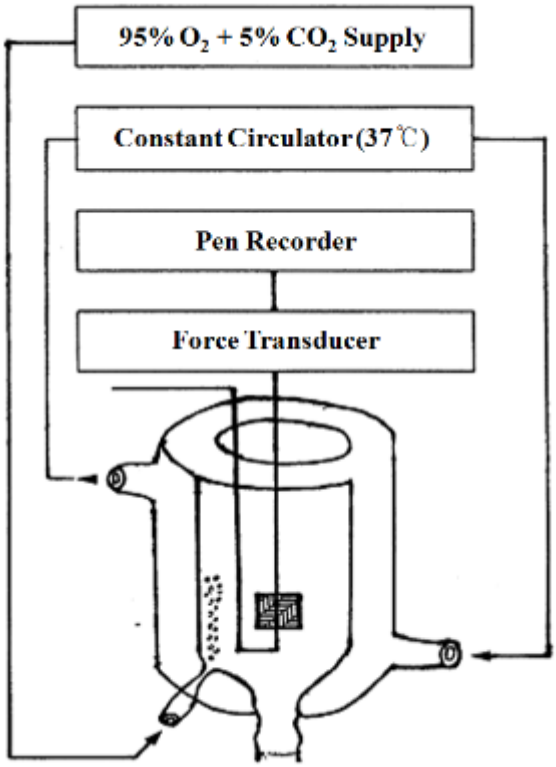


Fig 2. A schematic representation of the isometric contraction recording system with a vertical chamber. The chamber (25㎖) was maintained at 37℃ with temperature-regulated circulator and aerated with 95% O_2 and 5% CO_2.

e. Drugs and their sources

The following drugs were used: SFPE 4, EF-SFPE 4, phenylephrine hydrochloride, potassium chloride, 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 gram.

f. Statistical analysis

The statistical significance between groups was determined by the Student's 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 (SEM). The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray (1987).

3. Influence of SFPE4 and EF-SFPE 4 on blood pressure of normotensive rat and spontaneously hypertensive rats

a. Experiments of rats

Mature male Sprague-Dawley rats, weighing 200 to 300 g, were used in the experiment. On the day of experiment, a rat was anesthetized with thiopental sodium (50 ㎎/㎏) intraperitoneally, and was maintained with urethane (0.5 g/㎏) subcutaneous. The thorax was opened by a midline incision, and the heart and surrounding area were exposed by placing three hook retractors. 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 36.5℃ by a thermostatically controlling blanket and heating lamp throughout the course of the experiment.

b. Measurement of blood pressure

In order to observe the change of arterial pressure, one of the common carotid arteries was catheterized with polyethylene tubing (21G). The artery tubing was filled with heparin solution (400 IU/㎖) to prevent the blood coagulation during the experiment. The tubing was connected to a pressure transducer (Gould Co., USA) and pulse of mean arterial blood pressure was recorded on a biological polygraph (Grass Co., USA) continuously. At this time,

the signals from polygraph were recorded on the computer through PowerLab.

c. Drug administration

Another cannulation with polyethylene tubing (OD 0.61㎜) was made into a femoral vein for the administration of drugs and supplemental anesthetic agents as needed to maintain light surgical anesthesia. After exposing both side of femoral vein, polyethylene tube was inserted and fixed. One femoral vein was used to the oneshot drug infusion route and the other was used to the continuous infusion of F-SFPE 4 route. The oneshot infusion of drug was less than 150 ㎕ and the continuous infusion pace of F-SFPE 4 was less than 0.013 ㎖/min through using Infusion pump (Harvard).

d. Drugs and their sources

The following drugs were used: SFPE 4, EF-SFPE 4, norepinephrine bitartrate (Sigma Chemical Co., USA), thiopental sodium and heparin sodium (Daehan Choongwae Pharm. Co., Korea). Drugs were dissolved in 0.9% saline solution required except norepinephrine, which was dissolved in 0.9% acid saline (pH 4.0). Concentrations of all drugs used are expressed in terms of molar base and gram. Especially F-SFPE 4 infusion liquid was made after the rat considered (X ㎍/㎏/min).

e. Analysis of data

Changing blood pressure was checked after the experiment through PowerLab recorded on the computer. After we measured off changing value of the blood pressure (㎜Hg), the before or after drug delivery was done a comparative analysis. The experiment grades was indicated the average and standard error. Significance test was synthetically indicated by performing the Students' " t"-test and considering $P<0.05$ and less.

III. RESULTS

A. Purification of fibrinolytic active compound from SFPE

1. Isolation of fractions from SFPE

a. Isolation of fractions from SFPE 4 and fibrinolytic activity assays

(1) Extraction and purification of fibrinolytic activity form SFPE4

As summarized in Scheme 1, the fibrinolytic activity was purified from SFPE 4 through the following step: To know about the disjunct fibrinolytic activity, the experiment was carried out. After dividing H_2O, EtOAc and BuOH, we found that EtOAc was eluted.

Fibrinolytic activity of the F-SFPE 4 was assayed using fibrin plates. All sample were experimented as concentration of 50 ㎎/㎖. 20 ㎕ of each sample was carefully placed on fibrin plate. The plate was incubated for 30 min, 1 hour, 2 hours, and 3 hours at 37℃ and the diameter of the lytic circle was measured. After 30 min incubation, lytic circle formed by H_2O, EtOAc and BuOH on fibrin plate were 5, 10 and 6.5 mm respectively. At after 1 hour, the circles were 11, 15 and 12 mm respectively. In 2 hours of incubation, lytic circle of EtOAc and BuOH were 19 and 14 mm respectively, but H_2O fraction disappeared fibrinolytic activity. And, in 3 hours of incubation, lytic circle of EtOAc and BuOH were 19 and 14 mm respectively (Table 2).

Fraction	Effect is time dependent			
	30 min	1 hour	2 hours	3 hours
EtOAc fr.	10	15	19	21
BuOH fr.	6.5	12	14	16
Water fr.	5	11	-	-
SFPE 4	5	11	13	17
H_2O:DMSO=5:1*	-	-	-	-
Streptokinase**	-	6	9	10

(diameter: mm)

Table 2. Inhibition of fibrinolytic activity by ethylacetate, butanol and water fraction of primary selected SFPE 4.

* SFPE 4 fractions resolved by H_2O:DMSO=5:1 solvent.

(negative control)

** Streptokinase (positive control) is produced in the blood to break down the major constituent of blood clots fibrin, therefore dissolving clots once they have fulfilled their purpose in stopping bleeding.

(2) Column chromatography of EF-SFPE 4

EtOAc fraction was fractionated by silica gel column chromatography (10×13 ㎝; 63-200 ㎛ particle size) using a gradient of *n*-hexane:acetone (from 6:1 to 0:1), to yield nine combined fractions according to their TLC profiles. The fibrinolytic activity established by the gained nine fractions was measured by the fibrin plate method. 20 ㎕ of each sample was carefully placed on fibrin plate. Figure 3 is shown results after 3 hour incubation of F1-F9 in fibrin plate. As a result of the measurement, it was fibrinolytiv activity contained plentifully in F5 and F6.

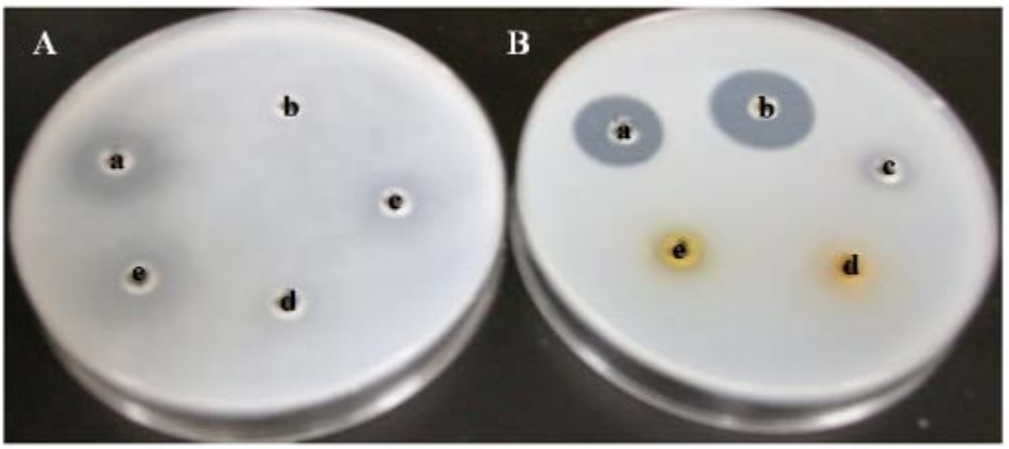


Fig 3. Lytic circles after 3 hours incubation of SFPE 4 F1-F9 in fibrin plate. A, SFPE 4 and Fraction F1-F4; lane a, SFPE 4; lane b, Fraction F1; lane c, Fraction F2; lane d, Fraction F3; lane e, Fraction F4; B, Fraction F5-F9; lane a, Fraction F5; lane b, Fraction F6; lane c, Fraction F7; lane d, Fraction F8; lane e, Fraction F9.

(3) Analysis of fibrinolytic activity compounds contained in F5 and F6

The partial information of the purified fractions F5.1-F5.5 and F6.1-F6.3 was gained by TLC analysis which is MeOH:H_2O (3:2) was used as a developing solvent (Fig. 4). When the fibrinolytic activity was measured by the fibrin plate method, we found that F6.1 fractions contain the fibrinolytic activity. The results of TLC chromatogram about fraction containing fibrinolytic activity showed that the bright pinkish red spot in the plate might be related with active fraction. Because the fibrinolytic activity of F6.1 was higher than others, the HPLC system was used for the isolation of fibrinolytic active compounds from fractions F6. The prominent perks were newly observed in polar elution part (retention time 17.8 min) on HPLC chromatogram of the F6 (Fig. 5, A) in comparison with that of F5 (Fig. 5, B). This result and the TLC data supported that isolated compound **1** was with fibrinolytic activity produced by F6 .

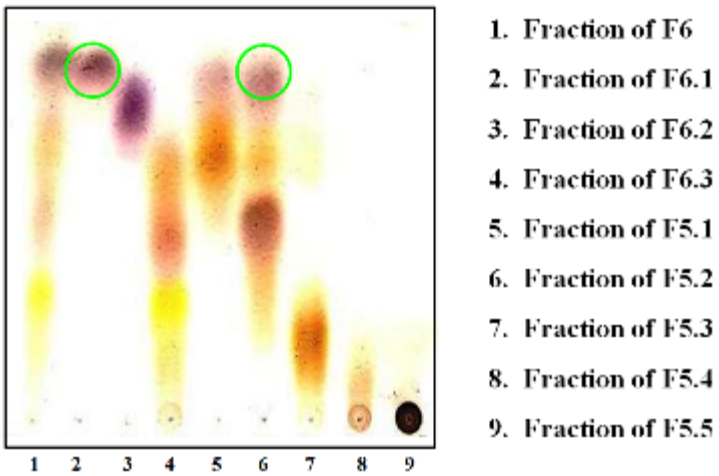


Fig. 4. TLC patterns of fraction F5 and F6 prepared of SFPE 4.
Developing solvent, MeOH:H_2O = 3:2 (v/v)

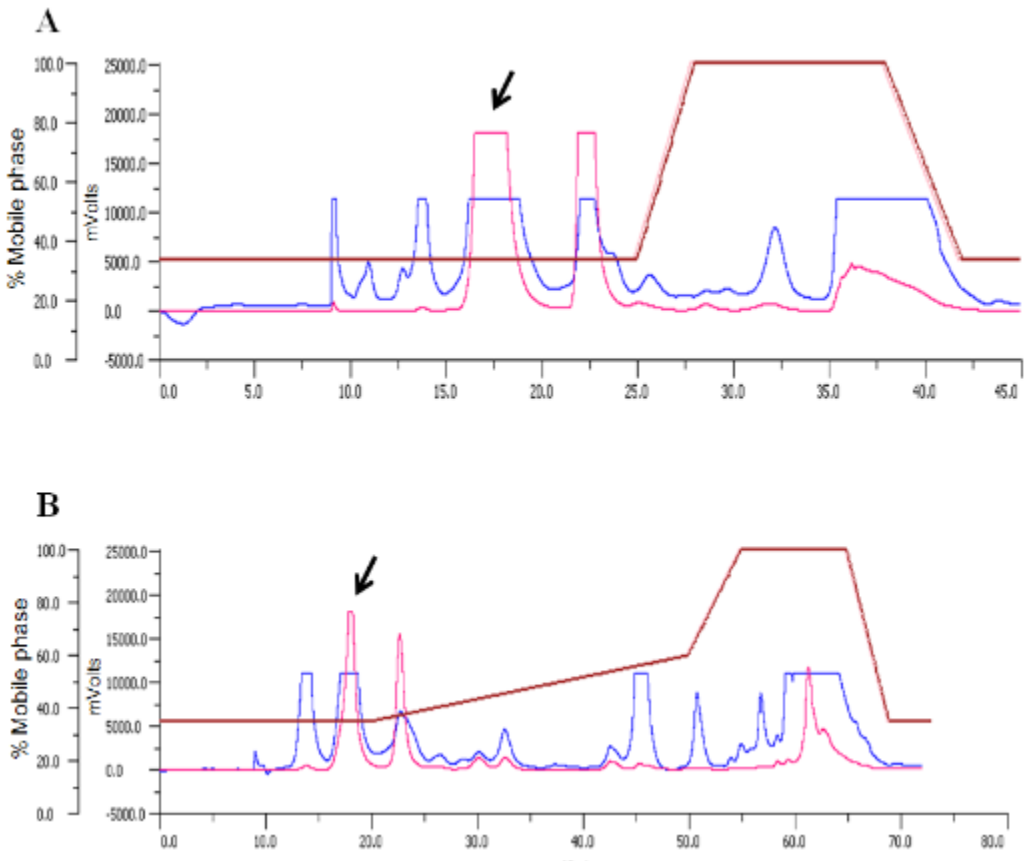


Fig 5. HPLC separation profile of F6 (A), and F5 (B). The wavelength for detection was tested at 205(blue peak) and 254(bright pink peak), where the compounds showed the maximum absorption as measured. HPLC condition : ODS-H80 column, flow rate : 3 ㎖/min, injection volumn : 120 ㎕.

(4) The chemical structure of protocatechuic acid isolated from SFPE 4

The chemical structure of purified compound F6.1 was identified by ^{1}H NMR and ^{13}C NMR spectroscopy (see Table 3). The ^{1}H NMR (500 MHz) data suggested that compound 1 included not only the chemical shifts and coupling patterns [δ_H 7.49 (d, J=7.5Hz), and 6.91(d, J=7.5Hz)]protons, but also coupling patterns of singlet proton at δ_H 7.55 (brs) (Fig. 6). These data supported that the structure of substance has a benzene ring. In the ^{13}C NMR spectrum, seven carbon signals including one carboxyl carbon δ_C 168.2 and six carbons [δ_C 150.7 (C-4), 145.5 (C-5), 123.8 (C-2), 122.9 (C-1), 117.5 (C-6), and 115.8 (C-3)] were measured (Fig. 7). By the comparison of the figures of literature between chemical-physico properties and ^{13}C NMR data, we confirmed that the structure of compound F6.1 was 3, 4-dihydroxybenzoic acid (protocatechuic acid) (Fig. 8).

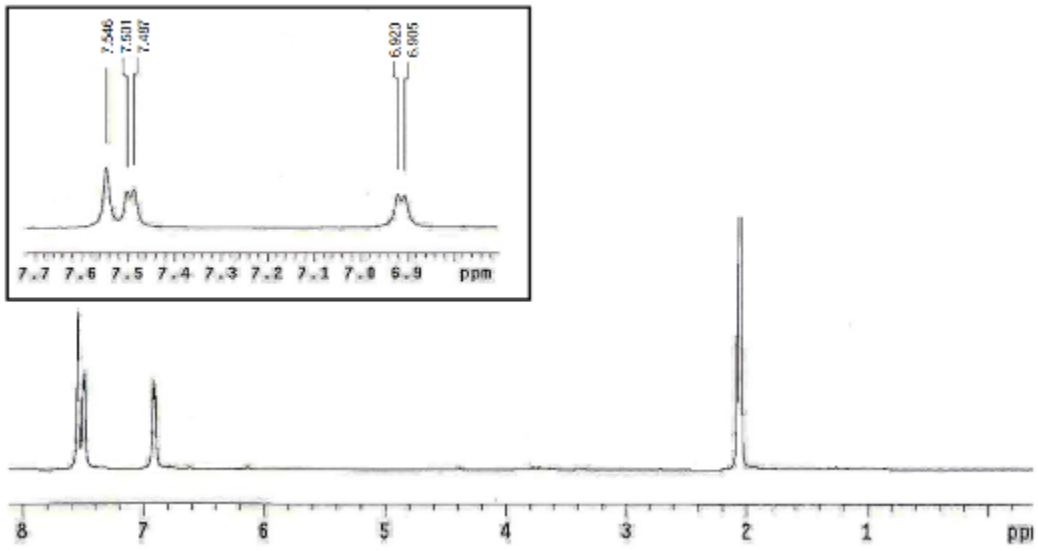


Fig 6. ^{1}H NMR spectrum of compound 1 isolated from SFPE 4.

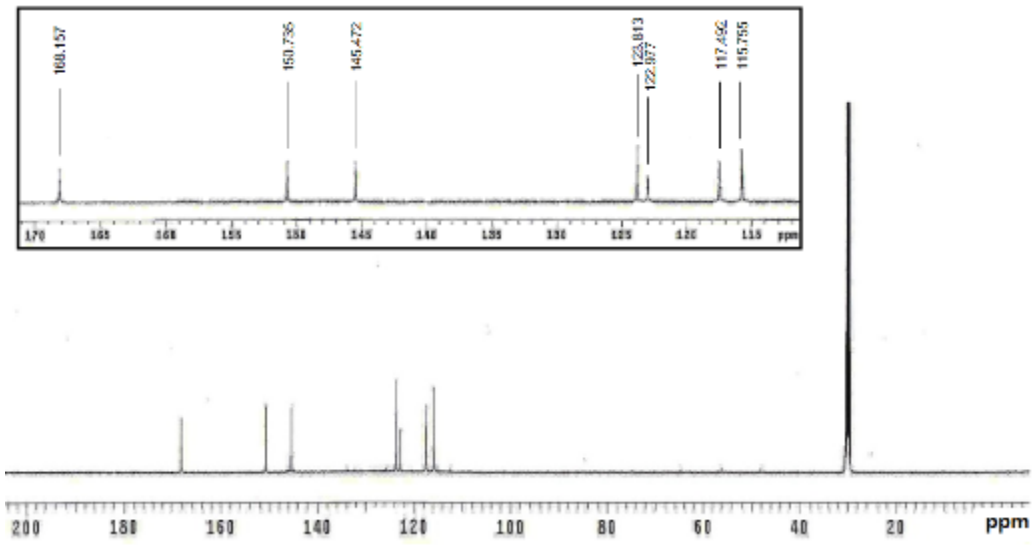


Fig 7. ^{13}C NMR spectrum of compound 1 isolated from SFPE 4.

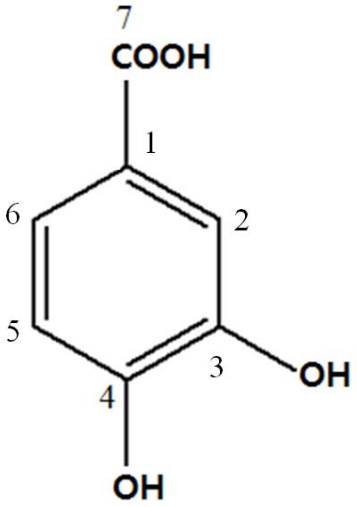


Fig 8. Chemical structure of 3, 4−dihydroxybenzoic acid isolated from F6.1 of SFPE 4. Formula: $C_7H_6O_4$, Exact Mass: 154.0266, m/e: 154.0266 (100.0%), 155.0300 (7.6%).

Position	δ_H mult. (J in Hz)	δ_C
1		123.8
2	7.55 brs	122.9
3		117.5
4		145.5
5	6.91 d (7.5)	150.7
6	7.49 d (7.5)	115.8
7		168.2

Table 3. ^{1}H NMR and ^{13}C NMR spectral data of compound **1**.

b. Isolationof of compound from SFPE 10 and fibrinolytic activity

(1) Extraction and purification of fibrinolytic activity form SFPE 10

As summarized in Scheme 2, the compound with fibrinolytic activity was purified from SFPE 10 through the following step: To know about the disjunct fibrinolytic activity, the experiment was carried out. After dividing to H_2O, EtOAc and BuOH, we found that BuOH layer has strong activity on fibrinolytic effects.

Fibrinolytic activity of the F-SFPE 10 was assayed using fibrin plates. All sample were experimented as concentration of 10 ㎎/㎖. 20 ㎕ of each sample was carefully placed on fibrin plate. The plate was incubated for 1 hour, 3 hours, and 5 hours at 37℃ and the diameter of the lytic circle was measured. After 1 hour incubation, lytic circle formed by H_2O, EtOAc and BuOH on fibrin plate were 12.5, 13 and 15 mm respectively. At after 3 hour, the circles were 14, 17 and 20 mm respectively. In 5 hours of incubation, lytic circle of H_2O, EtOAc and BuOH were 15.5, 22 and 25 mm respectively (Table 4).

(diameter: mm)

Fraction	Effect is time dependent		
	1 hour	3 hours	5 hours
EtOAc fr.	13	17	21
BuOH fr.	15	20	25
Water fr.	12.5	14	15.5
SFPE 10*	14	17	22
Streptokinase**	6.5	11	13

Table 4. Inhibition of fibrinolytic activity by ethylacetate, butanol and water fraction of primary selected SFPE 10.

* SFPE 10 was not resolved.

** Streptokinase was positive control.

(2) Analysis of fibrinolytic activity by compounds contained in FB1

The BuOH-soluble fraction (250 ㎎) was found to be most active among the solvent fraction. Fraction BuOH was applied to Sephadex LH-20 column chromatography (5 × 30 ㎝) using MeOH as the eluting solvent to give three fractions (FB1 – FB3). The partial information of the purified fractions FB1, FB2, and FB3 was TLC analysis with MeOH:H_2O (1:2) as a developing solvent (Fig. 9). When we measured the fibrinolytic activity of each fraction by the fibrin plate method. We found that FB1 fraction contains the most strong fibrinolytic activity. For the confirming of compound fibrinolytic activity from fractions FB1, HPLC system used for purification of active compound. The prominent perks were newly observed in polar elution part (retention time 10.0 min) on HPLC chromatogram of the F6 (Fig. 10). This result and TLC data supported that major peak was the compound with fibrinolytic activity produced by FB1.

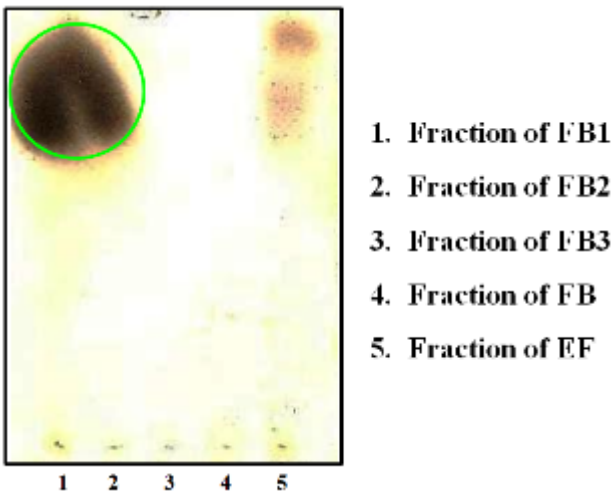


Fig. 9. TLC patterns of fraction FB and F5 prepared of SFPE 10.
Developing solvent, MeOH:H_2O = 1:2 (v/v).

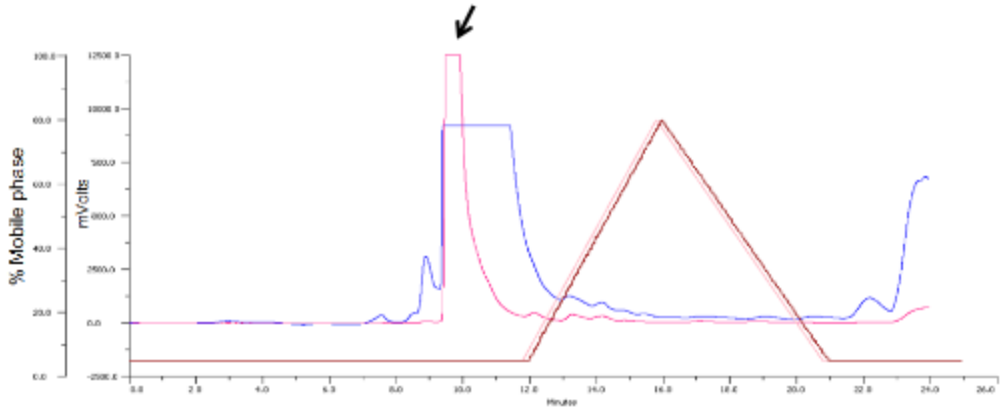


Fig 10. HPLC separation profile of FB from SFPE 10.

The wavelength for detection was tested at 205(blue peak) and 254(bright pink peak). HPLC condition : ODS-H80 column, solvent MeCN in H_2O containing 0.1% formic acid (0-13, 21-25 min: 5% MeCN and 16-20 min: 80% MeCN), flow rate: 3 ㎖/min, injection volumn: 130 ㎕.

(3) The chemical structure of shikimic acid isolated from SFPE 10

To confirm the chemical structure of isolated compound, the spectrum of ^{1}H NMR (Fig. 11) and ^{13}C NMR (Fig. 12) spectra were measured (^{1}H NMR and ^{13}C NMR data, see Table 5). In the ^{1}H NMR (300 MHz) spectrum, compound 2 showed ten carbon signals including three hydroxyl protons [δ_H 3.67 (1H, m), 3.95 (1H, td), and 4.43(1H, dd)], and singlet proton at δ_H 6.76 (1H, t), and peaks of doublets of doublet at δ_H 2.14 and 2.65 (2H, dd). In the ^{13}C NMR spectrum, chemical shifts of carbon signals including one carboxyl carbon δ_C 170.0 and six carbons [δ_C 138.8 (C-2), 130.7 (C-1), 72.7 (C-4), 68.4 (C-3), 67.3 (C-5), and 31.7 (C-6)] were determined. The chemical structure of compound 2 was determined to be shikimic acid by comparing the physicochemical properties and spectroscopic data with ^{1}H and ^{13}C NMR spectra (Fig. 13).

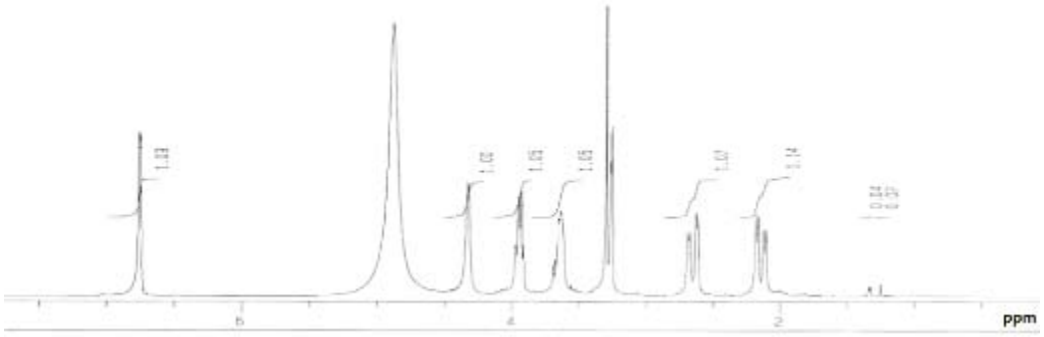


Fig 11. [1]H NMR spectrum of compound **2** isolated from SFPE 10.

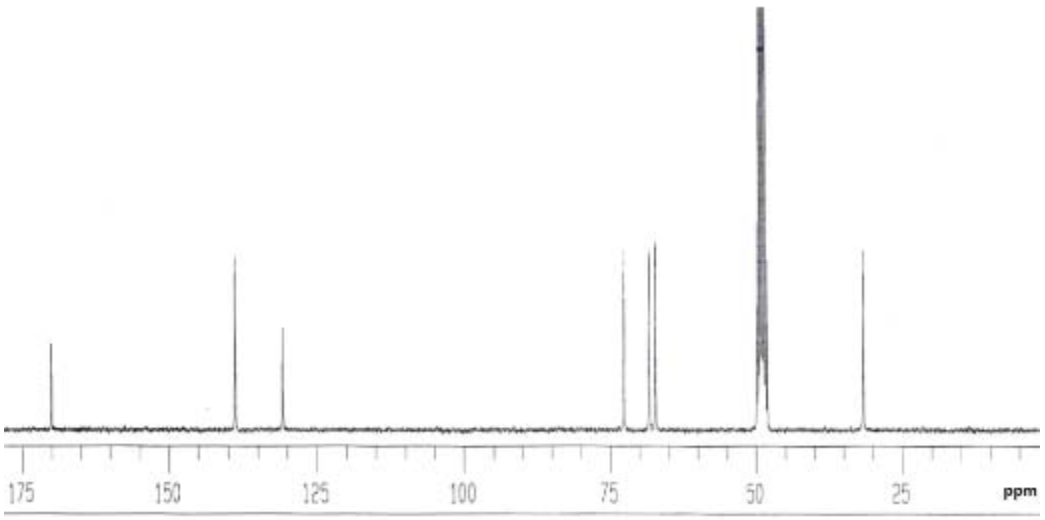


Fig 12. [13]C NMR spectrum of compound **2** isolated from SFPE 10.

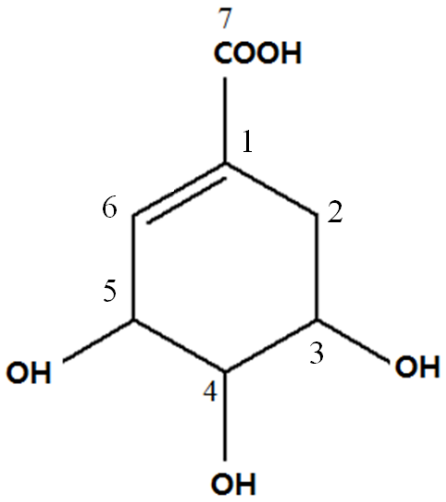


Fig 13. Chemical structure of shikimic acid isolated from FB1 of SFPE 10. Formula: $C_7H_{10}0_5$, Exact Mass: 174.0528, m/e: 174.0528 (100.0%), 155.0562 (7.6%), 176.0571 (1.0%).

Position	δ_H mult. (J in Hz)	δ_C
1	6.76 t	130.7
2		31.7
3	2.14 dd (18.0, 6.0) 2.65 dd (18.0, 6.0)	67.3
4	3.67 m	72.7
5	4.43 dd	68.4
6	3.95 td	138.8
7		170.0

Table 5. ^{1}H NMR and ^{13}C NMR spectral data of compound 2.

2. Identification of containing fibronolysis by microorganism from SFPEs

To identify and characterize microbial strains from SFPEs, we performed microbial strains screening. Several strains of bacteria and yeast were appeared in the stored extract without their inoculation. The fibrinolytic activity was purified by fibrin plate assay. 8 different microbial strains Isolates from SFPEs samples showed that No. 2 sample was strong fibrinolytic activity. Based on the extent of the activity, strains from an SFPEs sample was chosen for further characterization (Table 6).

No	Source	Fibrinolytic activity (mm)
1	SFPE 6	13
2	SFPE 2, and 4	18
3	PE	11
4	PE, SFPE 1, and 4	8
5	PE, SFPE 3, and 4	10
6	PE, SFPE 1	12.5
7	PE, SFPE 1	10
8	SFPE 2, and 3	10.5

Table 6. Fibrinolytic activities of 8 preliminary selected isolates.

For the identification of the fibrinolytic isolate, 16S rRNA and 26S rRNA primers was sequenced and compared using the BLAST program. There were altogether 8 different microbial strains isolated and identified (Fig. 14). The isolated strains were identified as *Ralstonia pickettii*, *Acetobacter tropicalis*, *Pichia galeiformis*, *Candida boidinii*, *Candida ernobii*, *Candida Karawaiewi*, *Saccharomyces cerevisiae*, and *Methylobacterium fujisawaense*. The PCR. The No. 2 isolate showed high identity to that of *Acetobacter tropicalis* (97%). Therefore, the novel fibrinolytic microorganism was referred to as *Acetobacter tropicalis* JY-2 (Fig. 15).

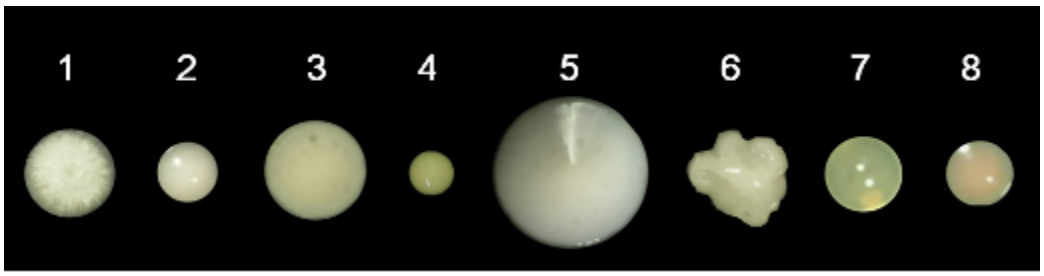


Fig 14. Structure of colonies of 8 different yeast strains isolated from the cultured medium. They were identified as 1. *Ralstonia pickettii*, 2. *Acetobacter tropicalis*, 3. *Pichia galeiformis*, 4. *Candida boidinii*, 5. *Candida ernobii*, 6. *Candida Karawaiewi*, 7. *Saccharomyces cerevisiae*, and 8. *Methylobacterium fujisawaense*.

5'-ATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCAATGC
CGCGTGTGTGAAGAAGGTTTTCGGATTGTAAAGCACTTTCGGCGGGGAC
GATGATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAG
CCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATGACTGGGCGTAAA
GGGCGTGTAGGCGGTTTGTACAGTCAGATGTGAAATCCCCGGGCTTAACC
TGGGAGCTGCATTTGATACGTGCAGACTAGAGTGTGAGAGAGGGTTGTG
GAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGGAAGAACACCGG
TGGCGAAGGCGGCAACCTGGCTCATGACTGACGCTGAGGCGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATG
TGTGCTGGATGTTGGGCAACTTAGTTGTTCAGTGTCGTAGCTAACGCGA
TAAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGCAGAACCTTACCAGGGCTTGTATGTGTAGGCTGTGTCCAGAGATGGG
CATTTCCCGCAAGGGACCTACAGCACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATC
TTTTAGTTGCCAGCATGTTTGGGTGGGCNCTCTAGAGAGACTGCCGGTG
ACAGCCGGAGANNTGGGGATGACGTCNGTCCTCATGGCCCTTATGTCCTG
GGCCTACNNNNGTGCTACATGGCGGTGANNNGGGGAAGCCTAGNATGCG
AANTCGNGCTGAT -3'

Fig 15. 16S rRNA BR1/BF2 region sequenced from *Acetobacter tropicalis* JY-2.

B. Blood pressure lowering action in the compounds from SFPE 4

1. Influence of F-SFPE 4 on catecholamine release in the isolated rat adrenal gland

a. Effects of F-SFPE 4 on CA secretion evoked by ACh, high K_+, from the perfused rat adrenal glands

After the perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, basal CA release from the isolated perfused rat adrenal glands amounted to 21 ± 2 ng for 2 min (n=10). Since in an *in vivo* study PCRW was shown to reduce CA Release in normotensive and hypertensive rats (Kee and Lim, 2007; Lim, 2008), it was attempted initially to examine the effects of F-SFPE4 itself on CA secretion from the perfused model of the rat adrenal glands. Secretagogues were given at 15 min-intervals. F-SFPE4 was present for 90 minutes after the establishment of the control release.

When ACh (3.52 mM) in a volume of 0.05 ㎖ was injected into the perfusion stream, the amount of CA secreted was 1284±25 ng for 4 min. However, in the presence of HF-SFPE 4 (60 ㎍/㎖) for 90 min, ACh-stimulated CA secretion was not affected, as compared to the control release of CA (1284±25 ng for 0~4 min). And, following the pretreatment with time-dependent of BF-SFPE 4 (60 ㎍/㎖), ACh-stimulated CA secretion was

inhibited to 94±2% ($P<0.05$) of the control after a 90-94 min period, although it was not initially affected by BF-SFPE 4 (60 ㎍/㎖). Meanwhile, in the presence of EF-SFPE 4 (60 ㎍/㎖) for 90 min, ACh-stimulated CA secretion was inhibited in time-dependent fashion. As shown in Fig. 16, in the presence of EF-SFPE 4 (60 ㎍/㎖), CA releasing responses were inhibited by 78±3% ($P<0.01$) of the control after a 60-94 min period. Also, it has been found thar depolarizing agent such as high potassium markedly stimulated the CA secretion (706±19 ng for 0-4 min). However, following the pretreatment with HF-SFPE 4 (60 ㎍/㎖), high K^+ (56 mM)-stimulated CA secretion was increased by 112±3% ($P<0.01$) of the control atl ast period (75-94 min). High K^+ (56 mM)-stimulated CA secretion after the pretreatment with BF-SFPE 4 (60 ㎍/㎖) was not affected. And, following the pretreatment with EF-SFPE 4 (60 ㎍/㎖), high K^+ (56 mM)-stimulated CA secretion was significantly inhibited to 78±3% ($P<0.01$) of the control after a 60-94 min period (Fig. 17). In the present study, ACh-stimulated and high K^+-stimulated CA secretion after the pretreatment with EF-SFPE 4 (60 ㎍/㎖) was significantly inhibited from the isolated perfused rat adrenal glands.

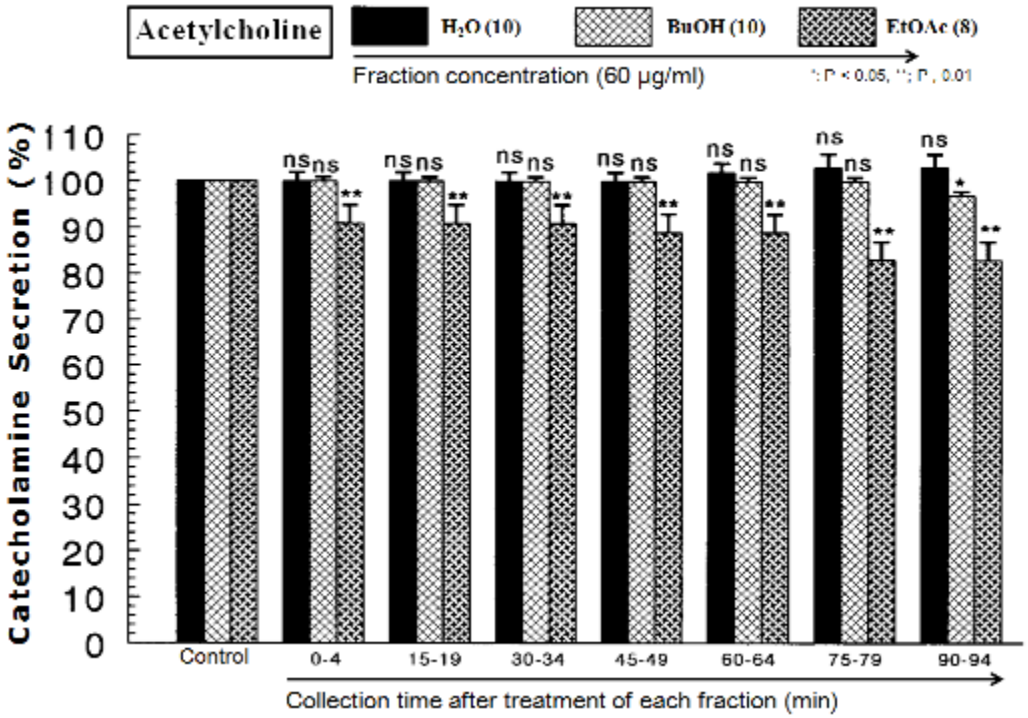


Fig 16. Dose-dependent effects of F-SFPE 4 on the secretory responses of CA evoked by ACh from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (3.52 mM) in a volume of 0.05 ㎖ was evoked at 15 min intervals after preloading with 60 μM of HF, BF and EF-SFPE 4 for 90 min as indicated at an arrow mark. Numbers in the parenthesis indicate number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean (SEM). Ordinate: the amounts of CA secreted from the adrenal gland (% of control for 4 min). Abscissa: collection time of perfusate (min). Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-pretreated group of F-SFPE 4. ACh-induced perfusate was collected for 4 min.

*: $P < 0.05$, **: $P < 0.01$. ns; Statistically not significant.

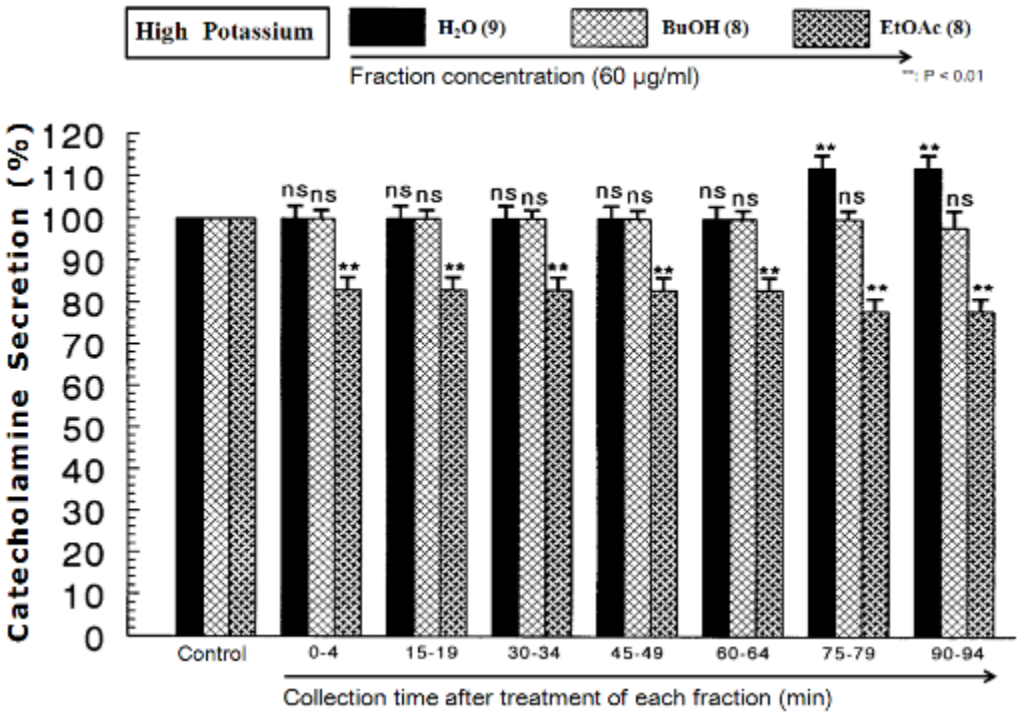


Fig. 17. Dose-dependent effects of F-SFPE 4 on the secretory responses of CA evoked by high K^+ from the isolated perfused rat adrenal glands. CA secretion by a single injection of K^+ (56 mM) was injected in a volume of 0.05 ㎖ at 15 min intervals after preloading with 60 μM of F-SFPE 4 for 90 min, respectively. K^+-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 16.

**: $P < 0.01$. ns; Statistically not significant.

b. Effect of EF-SFPE 4 on CA secretion evoked by ACh and Bay-K-8644 from the perfused rat adrenal glands

As shown in Fig. 14~15, it has also been shown that EF-SFPE 4 inhibits the CA secretory response evoked by ACh and high K^+ stimulation in the perfused rat adrenal gland. Therefore, in order to study the high dose effects of EF-SFPE 4 on the CA secretion, in the presence of high doses (20 and 180 μ M) of BF, and EF-SFPE 4, the CA secretory responses evoked by ACh and high K^+ stimulation were examined.

In the simultaneous presence of EF-SFPE 4 in the range of 20 ~ 180 μ M for 90 min, ACh-stimulated CA secretion was inhibited in concentration- and time-dependent fashion. As shown in Fig. 18, in the presence of EF-SFPE 4, CA releasing responses were inhibited by 71% ($P<0.01$) of the corresponding control release. ACh-evoked CA release in the presence of BF-SFPE 4, CA release for 90 min was also not changed for the first 79 min, but was later inhibited to 94% ($P<0.05$) of the corresponding control release in the last period, 90-94 min, in comparison to the control secretion (1397±28 ng for 4 min) from 8 glands (Fig. 19).

Since Bay-K-8644 is known to be a calcium channel activator, which enhances basal Ca^{2+} uptake (Garcia *et al.,* 1984) and CA release (Lim *et al.,* 1992), it was of interest to determine the effects of EF-SFPE 4 on Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands. Bay-K-8644 (10 μ M)-evoked CA secretion in the presence of EF-SFPE 4 (60 ㎍/㎖) was greatly blocked to 80~73% ($P<0.01$) of the control

at 60-94 min period as compared to the corresponding control release (491±14 ng for 0-4 min) from 12 adrenal glands as shown in Fig. 20.

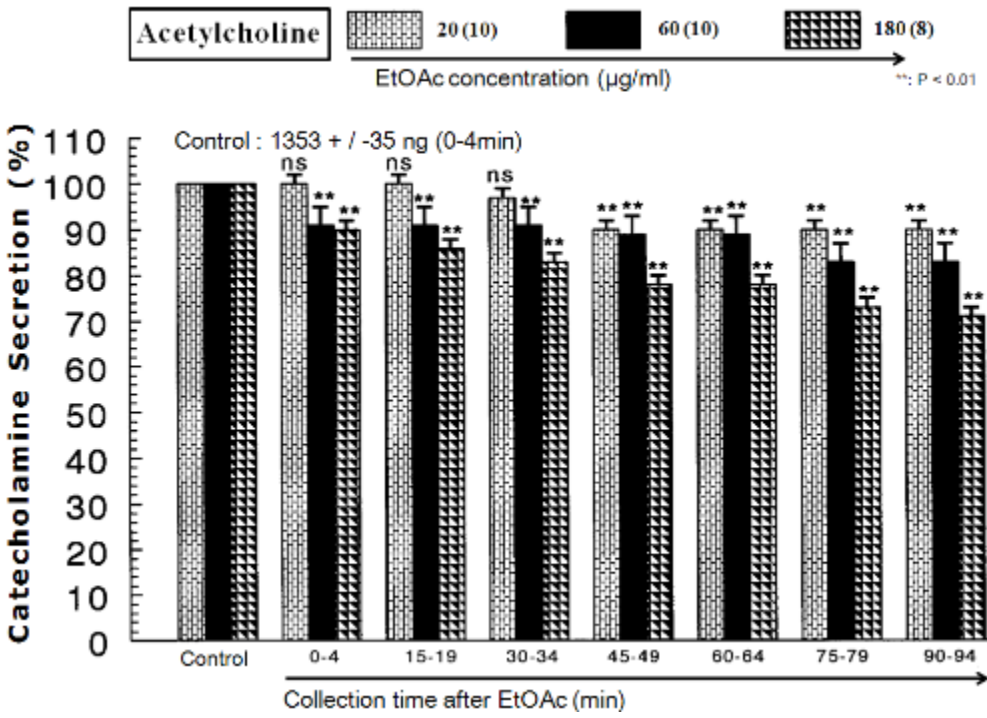


Fig 18. Dose-dependent effects of EF-SFPE 4 on the secretory responses of CA evoked by ACh from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (3.52 mM) in a volume of 0.05 ㎖ was evoked at 15 min intervals after preloading with 20, 60, and 180 μM of EF-SFPE 4 for 90 min as indicated at an arrow mark. Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-pretreated group of EF-SFPE 4. Other legends are the same as in Fig. 16. ACh-induced perfusate was collected for 4 minutes. *: $P < 0.01$. ns; Statistically not significant.

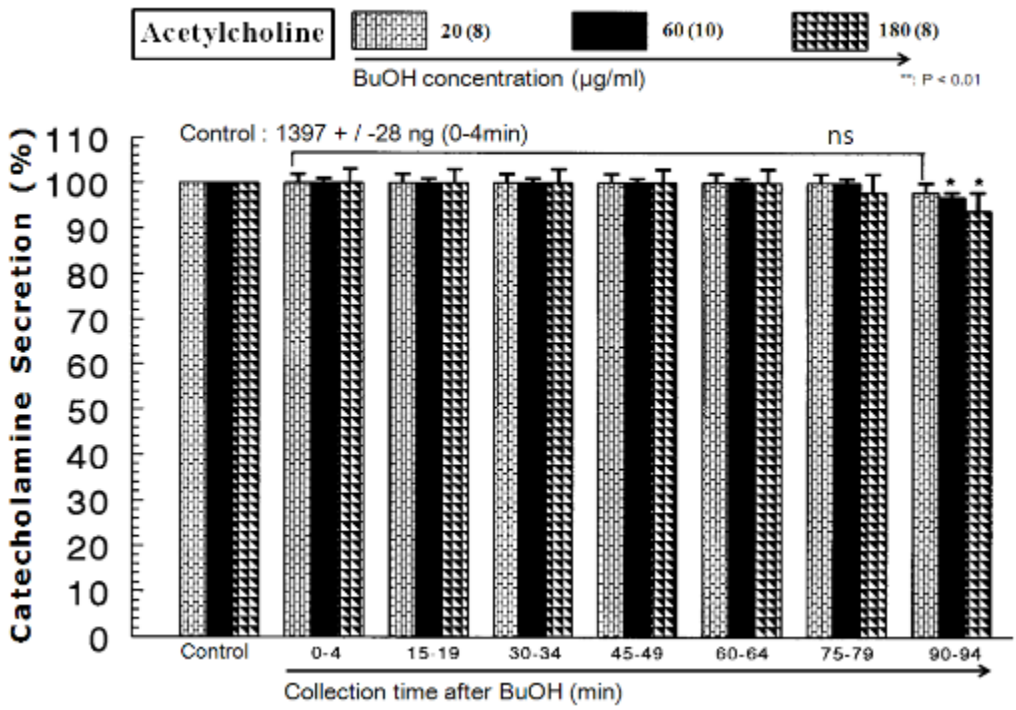


Fig 19. Dose-dependent effects of BF-SFPE 4 on the secretory responses of CA evoked by ACh from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (3.52 mM) in a volume of 0.05 ㎖ was evoked at 15 min intervals after preloading with 20, 60, and 180 μM of EF-SFPE 4 for 90 min as indicated at an arrow mark. Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-pretreated group of EF-SFPE 4. Other legends are the same as in Fig. 16.

*: $P < 0.05$. ns; Statistically not significant.

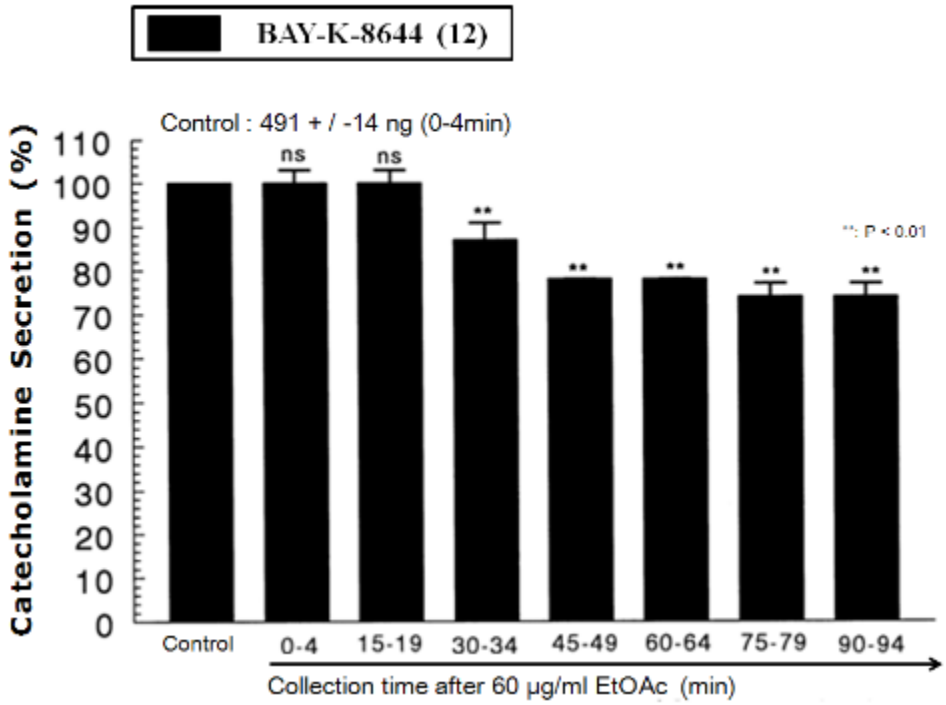


Fig 20. Time-course effects of EF-SFPE 4 on the CA release evoked by Bay-K-8644 from the rat adrenal glands. Bay-K-8644 (10 μM) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with EF-SFPE 4 (60 ㎍/㎖) for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-pretreated group of EF-SFPE 4. Other legends are the same as in Fig. 16.

*: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

2. Influence of SFPE 4 and EF-SFPE 4 on contractile responses of aortic strips isolated from rats

a. Effects of SFPE 4 on contractile responses induced by phenylephrine and high potassium (KCl) in the thoracic aortic strips of rat

The resting (basal) 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. Since in an *in vivo* study PCRW was shown to contractile responses induced by phenylephrine and high potassium in normotensive and hypertensive rats (Lim, 2008), it was attempted initially to examine the effects of SFPE 4 on contractile responses induced by phenylephrine and high potassium in the thoracic aortic strips of Rat.

When 10 μ M concentration of phenylephrine was administered into the aortic bath, its active tension amounted to 2.1±0.2 g from the resting tension level. In the presence of SFPE 4 at 360, 720 and 1440 ㎍/㎖, the contractile responses of phenylephrine (10 μ M) were dos cdependently reduced to 73±6% ($P<0.01$, n=8), 56±6% ($P<0.01$, n=5) and 52±2% ($P<0.01$, n=5) of the corresponding control response, respectively (Fig. 21 and Fig. 22). High potassium 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 concentrations of 56 mM, which is a membrane-depolarizing agent, caused an increase in aortic contraction (1.6±0.2 g). As shown in Fig. 23 and Fig. 24, high potassium (56 mM)-induced contractile responses after pre-loading with 360, 720 and 1440 ㎍/㎖ of SFPE 4 were 81±12% ($P< 0.01$, n=7), 68±12% ($P<0.01$, n=5) and 57±7% ($P<0.01$, n=5) of their corresponding responding control responses in a dose-dependent fashion.

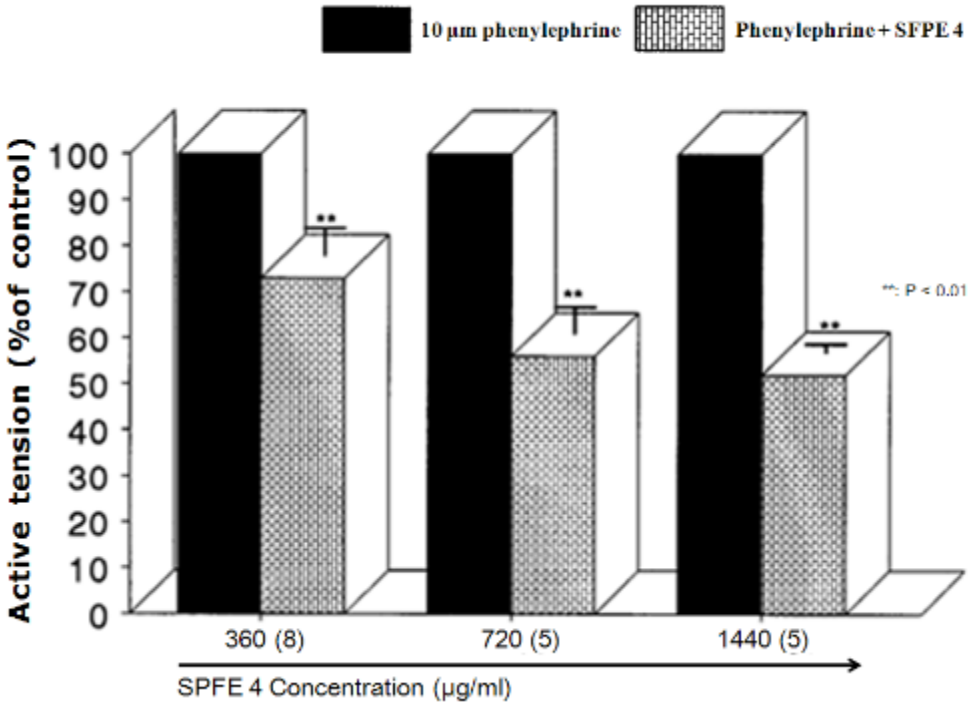


Fig 21. Influence of SFPE 4 on phenylephrine-induced contractile response in the isolated rat aortic strips. The contractile response was induced by adding 10 μM of phenylephrine after adaptation with normal Krebs solution. "CONTROL" and "AFTER" denote active tension induced evoked by phenylephrine before (CONTROL) and after adding 360, 720, and 1440 μg/㎖ of SFPE 4. Numerals in the parenthesis indicate number of experimental rat aortic strips. Vertical bar represent the standard error of the mean (SEM). Ordinate: the active tension (% of control). Abscissa: Concentrations of phenylephrine (10 μM). Statistical difference was obtained by comparing the control with the SFPE 4 -pretreated group.

**: $P < 0.01$. ns: Statistically not significant.

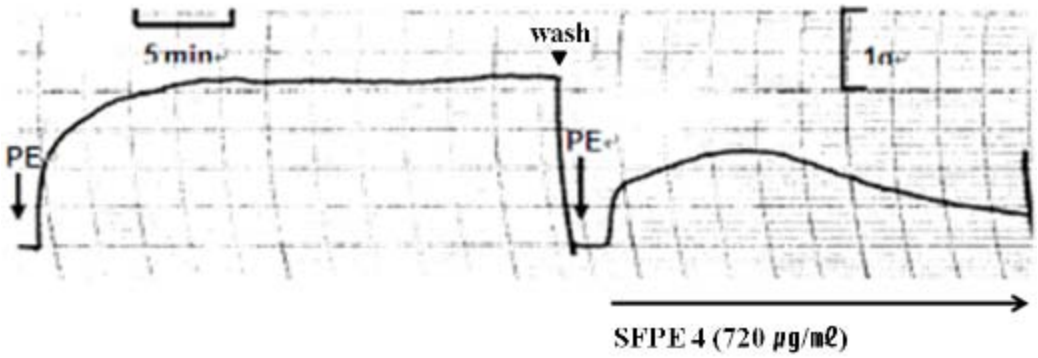


Fig 22. The typical tracing showing the inhibitory effect of SFPE 4 on phenylephrine-induced contractile response in the rat aortic strip.

Left: phenylephrine-induced contractile response (Control).

Right: phenylephrine- induced contractile response in the presence of SFPE 4 (720 ㎍/㎖). At arrow mark, the indicated dose of phenylephrine (10 μM) was added to the bath. The chart speed was 5 ㎜/min.

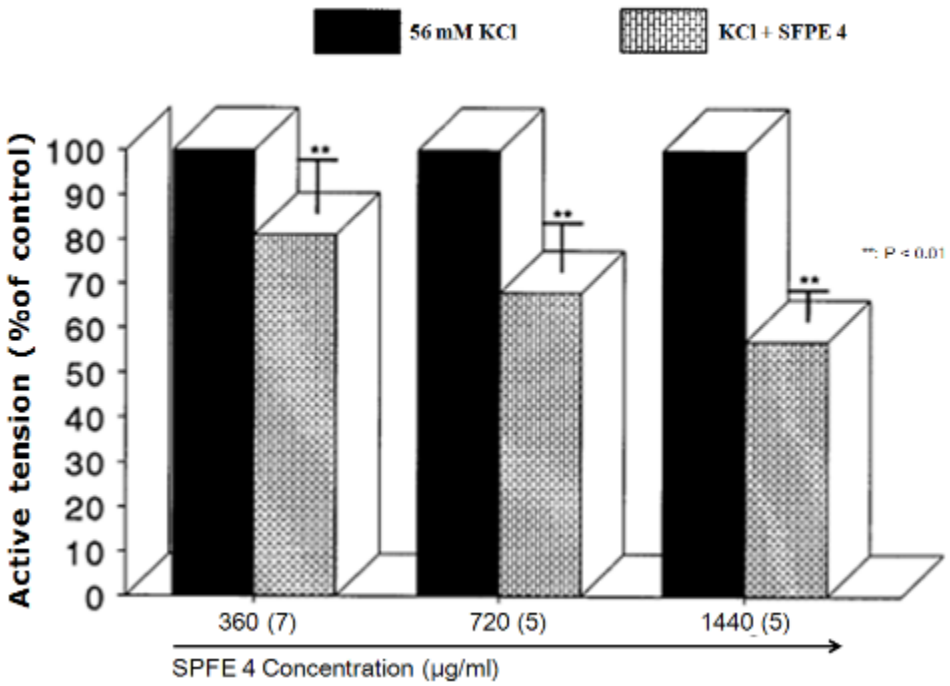


Fig 23. Influence of SFPE 4 on high potassium-induced contractile response in the isolated rat aortic strips. High potassium (56 mM) was added into the bath before and after pretreatment with 360, 720, and 1440 ㎍/㎖ of SFPE 4. Other legends are the same as in Fig. 21.

**: $P < 0.01$.

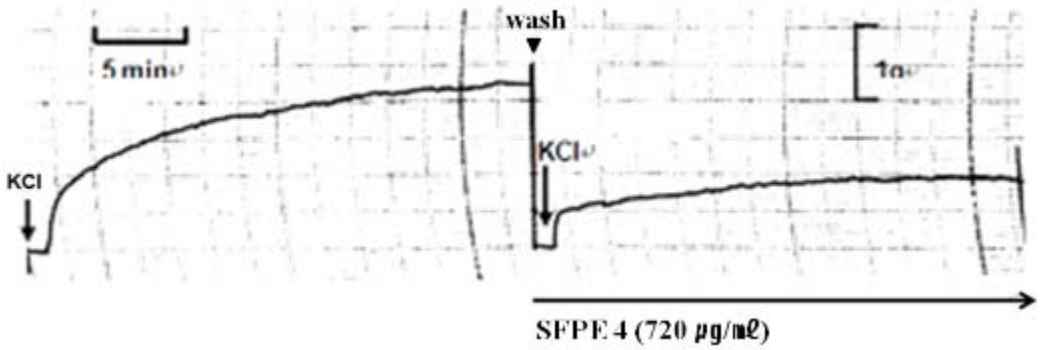


Fig 24. The typical tracing showing the inhibitory effect of SFPE 4 on high potassium-induced contractile response in the rat aortic strip.

Left: high potassium-induced contractile response (Control).

Right: KCl- induced contractile response in the presence of SFPE 4 (720 ㎍/㎖). At arrow mark, the indicated dose of KCl (56 mM) was added to the bath. The chart speed was 5 ㎜/min.

b. Effects of EF-SFPE 4 on contractile responses induced by phenylephrine and high potassium (KCl) in the thoracic aortic strips of rat

When 10 μ M concentration of phenylephrine was administered into the aortic bath, its active tension amounted to 2.1±0.3 g from the resting tension level. However, in the presence of EF-SFPE4 (400 ㎍/㎖), their active tensions were reduced to 42±5% ($P< 0.01$, n=6) of the control contractile responses (Fig. 25 and Fig. 26). As shown in Fig. 25 and Fig. 26, high potassium (56 mM) induced contractile responses after pre-loading with 400 ㎍/㎖l of EF-SFPE 4 were inhibited by 40±5% ($P<0.01$, n=6) of their corresponding control responses (2.0±0.1 g).

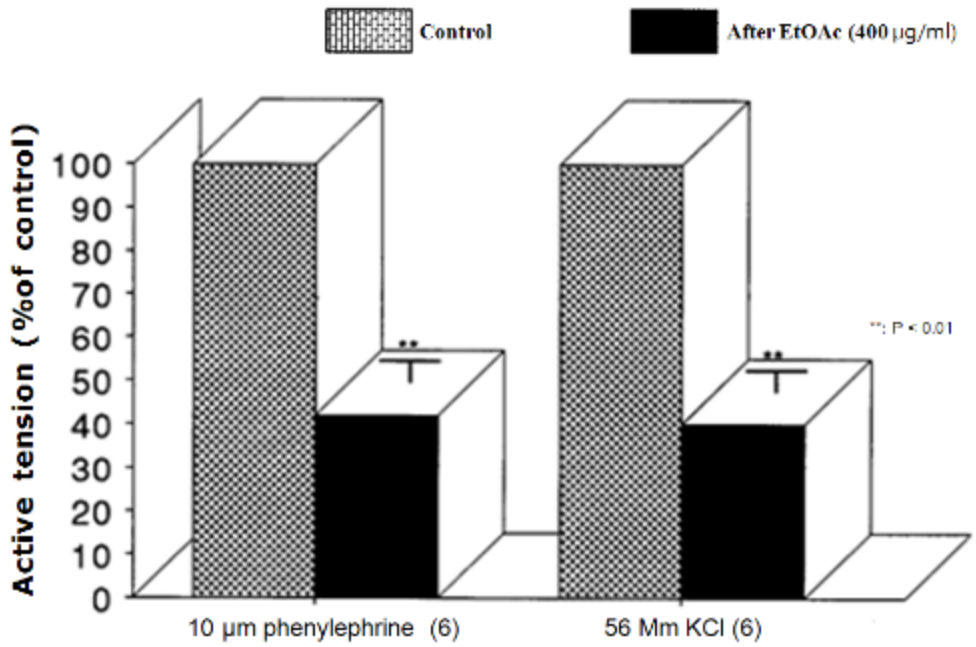


Fig 25. Influence of EF-SFPE4 on phenylephrine and high potassium-induced contractile response in the isolated in the isolated rat aortic strips. Phenylephrine (10 μM) and High potassium (56 mM) was added into the bath before and after pretreatment with 400 ㎍/㎖ of SFPE 4. Other legends are the same as in Fig. 17.

**: $P < 0.01$. ns: Statistically not significant.

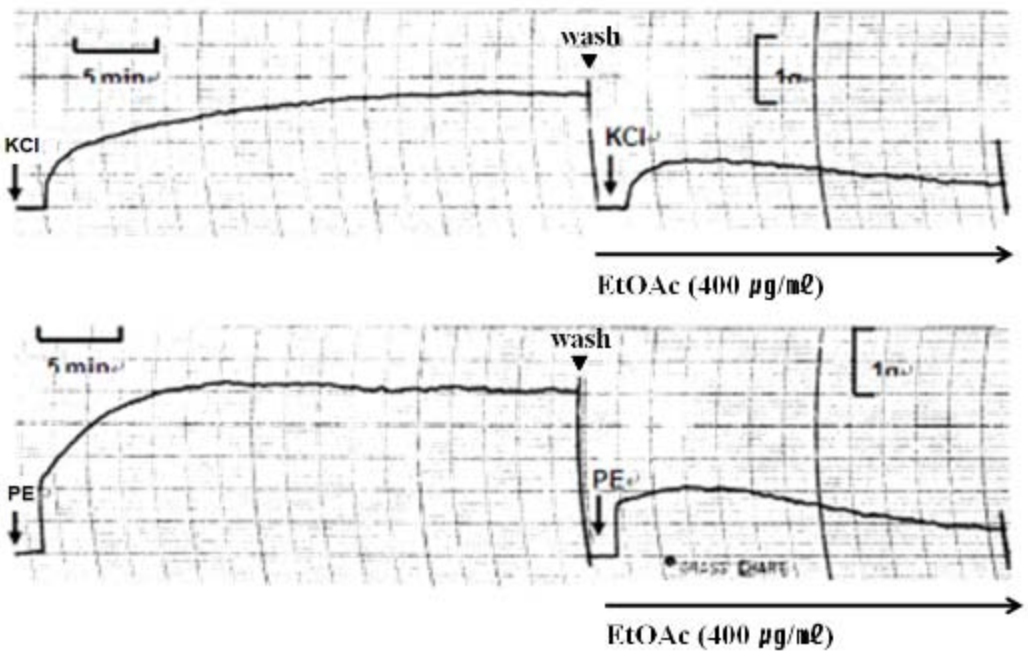


Fig 26. The typical tracing showing the inhibitory effect of EF-SFPE4 on phenylephrine and high potassium-induced contractile response in the rat aortic strip.

Left: phenylephrine and KCl-induced contractile response (Control). Right: phenylephrine and KCl-induced contractile response in the presence of SFPE 4 (720 ㎍/㎖). At arrow mark, the indicated dose of phenylephrine (10 μM) and KCl (56 mM) was added to the bath. The chart speed was 5 ㎜/min.

3. Influence of SFPE4 and EF-SFPE 4 on blood pressure of normotensive rat and SHRs

a. Influence of intravenous SFPE 4 and EF-SFPE 4 on norepinephrine (NE)-evoked pressor responses in the anesthetized normotensive rat

When cardiovascular parameters were stabilized for 30~60 min before the experimental protocols were initiated, the administration of physiological saline solution in a volume 0.2 ㎖ into a femoral vein did not cause any changes in both arterial blood pressure. Then, EF-SFPE 4 injected intravenously to the anesthetized normotensive rats produced a dose-dependent decrease in arterial blood pressure. In 6 rats, as shown in Fig. 27, intravenous doses of EF-SFPE 4 to 1.0, 3.0, and 10.0 ㎎/㎏ caused the dose-related reduction in arterial pressure responses to 4.8±0.3 ㎜Hg, 14.8±0.9 ㎜Hg and 23.7±1.3 ㎜Hg, respectively. Also, it was tried to test the effect of EF-SFPE 4 on NE-induced hypertensive responses in the anesthetized normotensive rats. In 8 normotensive rats, as shown in Fig. 28, norepinephrine at doses of 0,3, 1.0 and 3.0 ㎍/㎏ caused dose-dependent pressor responses of 14.6±2 ㎜Hg, 27±2 ㎜Hg and 47±3±1.7 ㎜Hg from the original baseline (126.1±17.2 ㎜Hg), respectively. After infusion of EF-SFPE 4 with a rate of 1 ㎎/㎏/30min, hypertensive responses of NE were inhibited to 7±1.2 ㎜Hg ($P<0.01$), 18.7±2 ㎜Hg ($P<0.01$) and 34±2.2 ㎜Hg ($P<0.01$) of control responses at the above same doses, respectively (Fig. 28 and 29). And, after increasing the of EF-SFPE 4 to 3 ㎎/㎏/30min, NE-evoked hypertensive responses at doses of

0,3, 1.0 and 3.0 ㎍/㎏ were significantly inhibited to 4±1 ㎜Hg (P<0.01), 12.4±1.8 ㎜Hg (P<0.01) and 20.7±2.4 ㎜Hg (P<0.01) of control responses at the above same doses, respectively, as shown in Fig. 30 and 31.

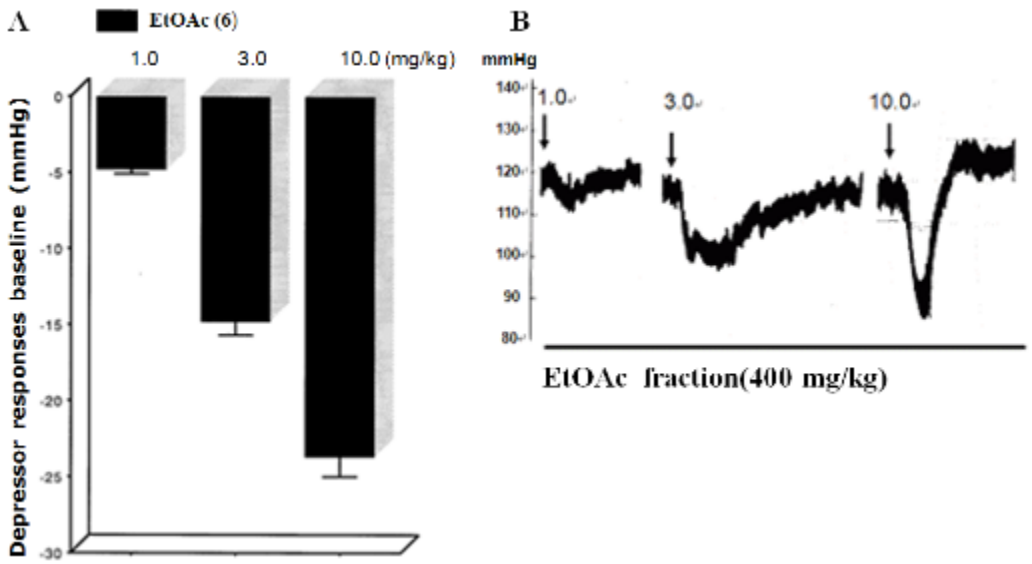


Fig 27. Effects of intravenous EF-SFPE 4 on arterial blood pressure in the anesthetized rats.

A: Influence of intravenous EF-SFPE 4 on arterial blood pressure in the thiopental-anesthetized rats. Ordinate: changes of arterial blood pressure in ㎜Hg from 6 rats. Abscissa: intravenous doses of EF-SFPE 4 in ㎍/㎏. B: The representative tracing of the intravenous EF-SFPE 4-induced depressor response in the thiopental-anesthetized rat. At arrow mark, the indicated doses (1.0, 3.0 and 10.0 ㎍/㎏) of EF-SFPE 4 were administered into a femoral vein at 30-60 min interval. The chart speed was 20 ㎜/min .

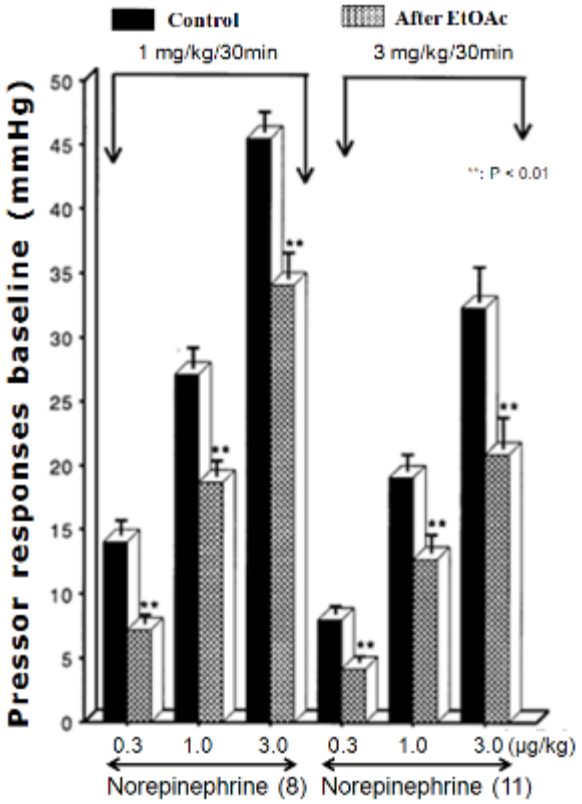


Fig 28. Influence of intravenous EF-SFPE 4 on NE-evoked pressor responses. Ordinate: Changes of blood pressure from baseline level in ㎜Hg. Abscissa: Intravenous doses of NE in 0.3, 1.0 and 3.0 ㎍/㎏. Vertical bar on the top of each column indicates standard error of mean. There was statistically significant difference in changes of NE-evoked pressor responses between before and after pretreatment with EF-SFPE 4. EF-SFPE 4 was infused into a femoral vein with a rate of 0.3, 1.0 and 3.0 ㎍/㎏/30 min after obtaining the corresponding control responses of intravenous NE.

**: P<0.01.

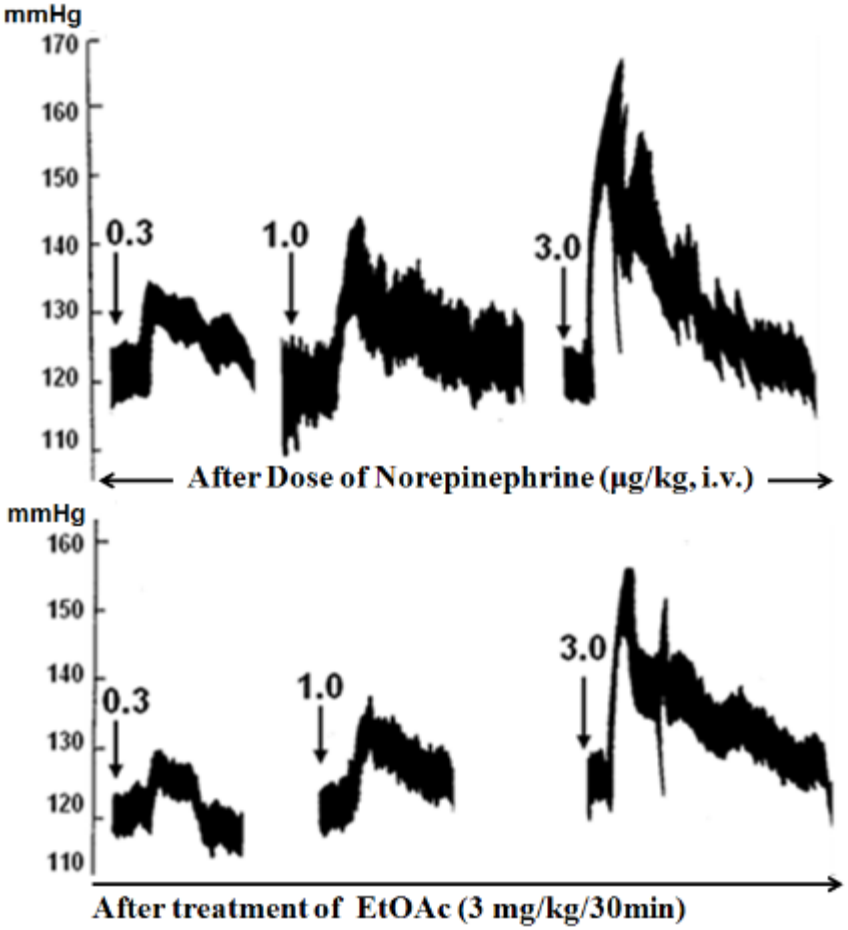


Fig 29. The representative tracing of EF-SFPE 4 effect on intravenous NE-induced pressor responses in the anesthetized rat. At arrow marks, the indicated doses (0.3, 1.0 and 3.0 ㎍/kg) of NE were administered into a femoral vein.

Upper panel: NE-induced hypertensive responses in a non treated rat. Lower panel: NE-induced hypertensive responses in a EF-SFPE 4 -pretreated rat. The chart speed was 10 mm/min.

In 10 rats, NE at doses of 0.3, 1.0, and 3.0 ㎍/㎏ caused dose-dependent pressor responses of 11.3±1.2 ㎜Hg, 21.4±1.8 ㎜Hg, and 34±2.6 ㎜Hg, respectively. However, after infusion of SFPE 4 with a rate of 3 ㎎/㎏/30 min, they were significantly depressed to 6.8±1 ㎜Hg ($P<0.01$), 14.5±1.5 ㎜Hg ($P<0.01$), and 23.3±12.6 ㎜Hg ($P<0.01$) at the above same doses, respectively (Fig. 30 and 31). And, after increasing the dose of SFPE 4 to 10 ㎎/㎏/30min, NE-evoked hypertensive responses at doses of 0,3, 1.0 and 3.0 ㎍/㎏ were significantly inhibited to 6.3±1.8 ㎜Hg ($P<0.01$), 11.5±1.5 ㎜Hg ($P<0.01$) and 16.2±2.7 ㎜Hg ($P<0.01$) of control responses at the above same doses, respectively, as shown in Fig. 30 and 31.

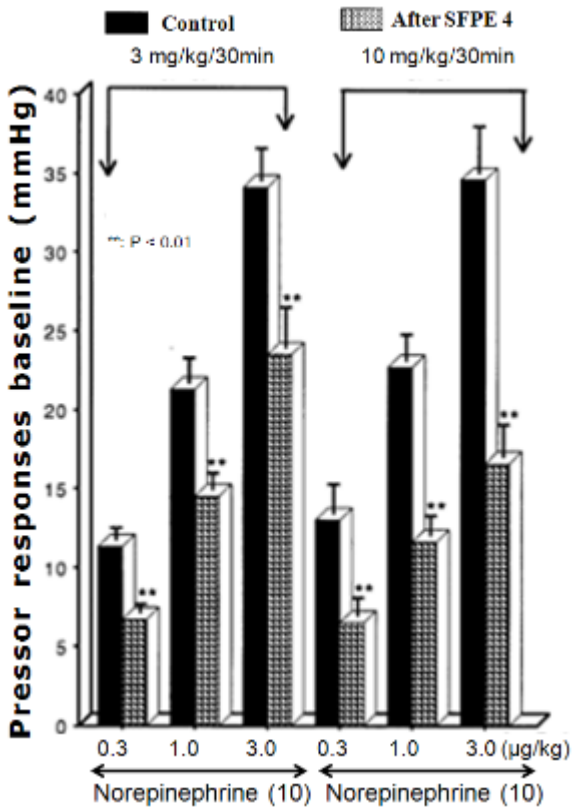


Fig 30. Influence of intravenous SFPE 4 on NE-evoked pressor responses. Ordinate: Changes of blood pressure from baseline level in ㎜Hg. Abscissa: Intravenous doses of NE in 0.3, 1.0 and 3.0 ㎍/㎏. Vertical bar on the top of each column indicates standard error of mean. There was statistically significant difference in changes of NE-evoked pressor responses between before and after pretreatment with SFPE 4. SFPE 4 was infused into a femoral vein with a rate of 0.3, 1.0 and 3.0 ㎍/㎏/30 min after obtaining the corresponding control responses of intravenous NE.

**: P<0.01.

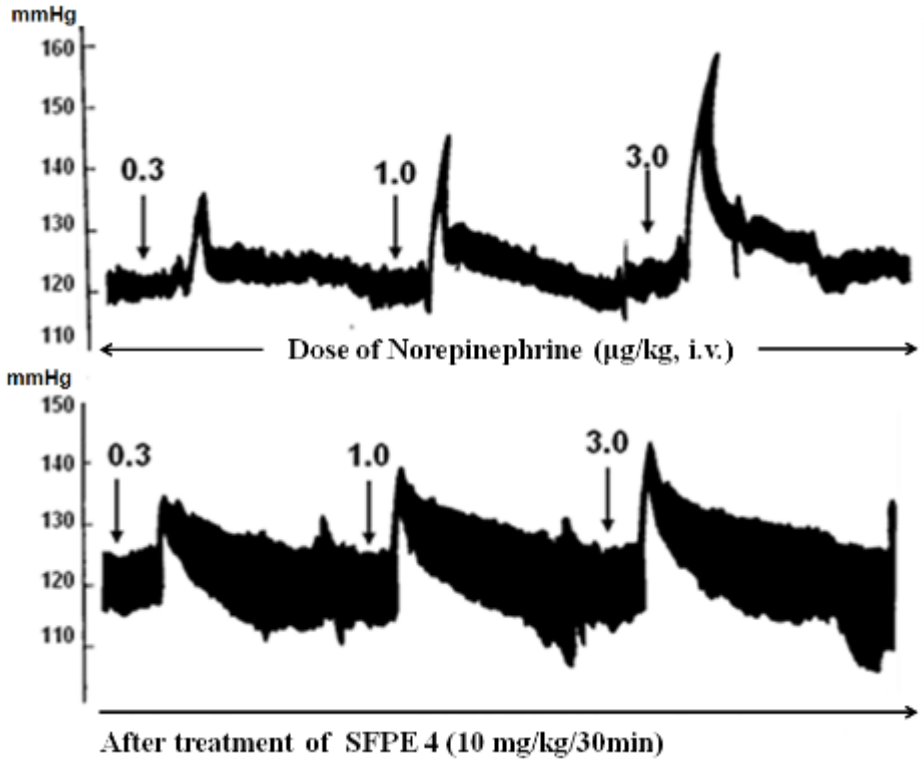


Fig 31. The representative tracing of SFPE 4 effect on intravenous NE-induced pressor responses in the anesthetized rat. At arrow marks, the indicated doses (0.3, 1.0 and 3.0 ㎍/㎏) of NE were administered into a femoral vein.

Upper panel: NE-induced hypertensive responses in a non treated rat. Lower panel: NE-induced hypertensive responses in a SFPE 4 -pretreated rat. The chart speed was 10 mm/min.

b. Influence of intravenous SFPE 4 on norepinephrine (NE)-evoked pressor responses in the anesthetized SHRs

Since SFPE 4 greatly inhibited phenylephrine-induced contractile response of the aortic strip, as shown in Fig. 2, it suggests that SFPE 4 might cause hypotension through the blockade of peripheral adrenergic α-receptors. It is also of interest to examine the effect of SFPE 4 on norepinephrine-evoked pressor responses.

In 6 SHRs, as shown in Fig. 18, norepinephrine at doses of 0,3, 1.0 and 3.0 μg/kg caused dose-dependent pressor responses of 16.5±0.3 ㎜Hg, 21.5±0.9 ㎜Hg and 28±0.6 ㎜Hg from the original baseline (182.5±18.5 ㎜Hg), respectively. After infusion of SFPE 4 with a rate of 3 mg/kg/30min and 10 kg/30min , hypertensive responses of norepinephrine were greatly inhibited to 57% ($P< 0.01$), 74% ($P< 0.01$) and 84% ($P< 0.01$) of control responses at the above same doses, respectively (Fig.32 and 33).

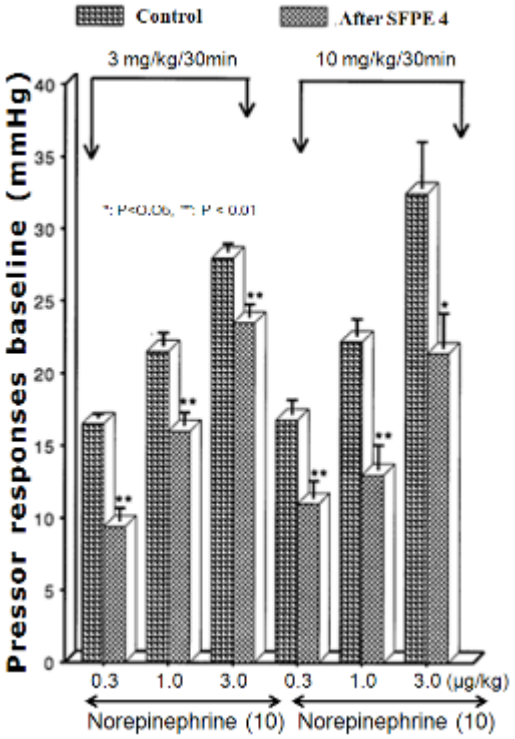


Fig 32. Influence of intravenous SFPE 4 on NE-evoked pressor responses in anesthetized SHRs. SFPE 4 (0.3, 1.0 and 3.0 ㎍/㎏/30 min, respectively) was given intravenously after obtaining the corresponding control responses of intravenous norepinephrine (0.3, 1.0 and 3.0 ㎍/㎏). Ordinate: changes of arterial blood pressure in ㎜Hg from 6 rats. Abscissa: intravenous doses of NE in ㎍/㎏. Vertical bars on each column indicate standard error of mean (SEM). There was statistically significant difference in changes of NE-evoked arterial pressor responses from pre-injection level before and after pretreatment of SFPE 4 (0.3, 1.0 and 3.0 ㎍/㎏/30 min). The originalbase-line of arterial blood pressure was 182.5±18.5 ㎜Hg.

*: P<0.05, **: P< 0.01. ns: Statistically not significant.

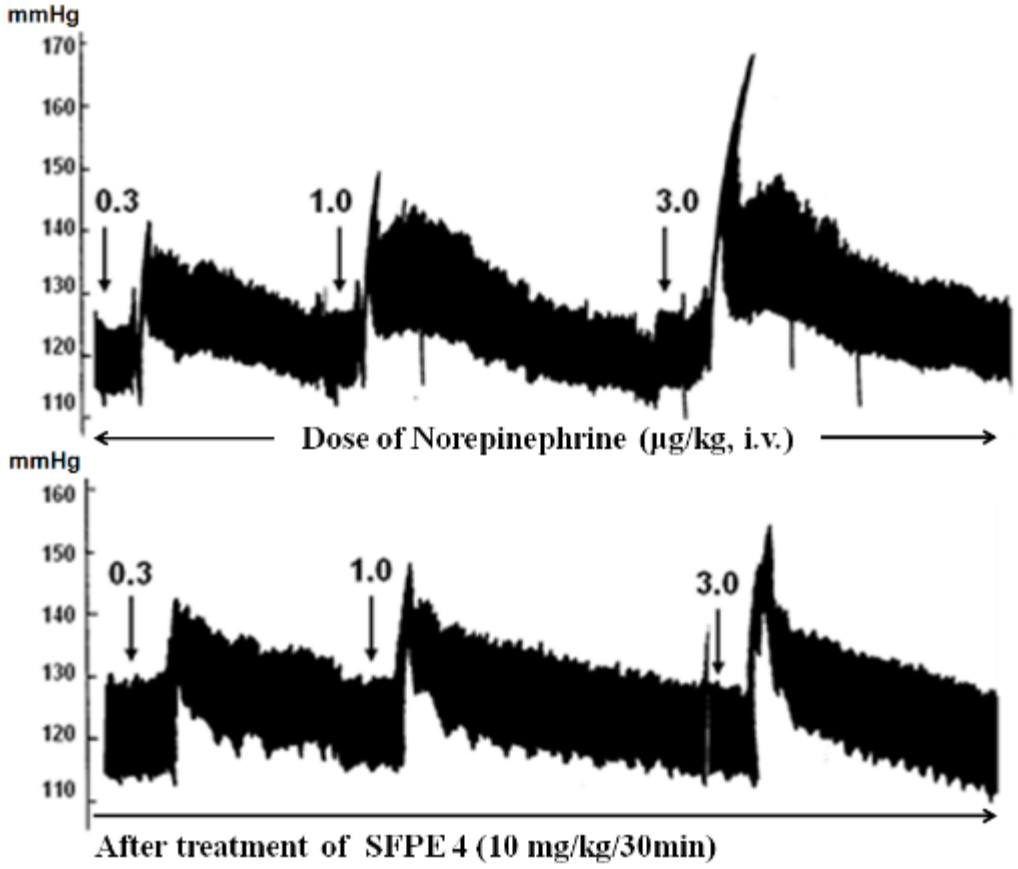


Fig 33. The representative tracing of SFPE 4 effect on intravenous NE-induced pressor responses in an anesthetized SHR. At arrow marks, the indicated doses (0.3, 1.0 and 3.0 ㎍/㎏) of NE were administered into a femoral vein. Upper: NE-induced hypertensive responses in a non-treated rat. Lower: NE induced hypertensive responses in a SFPE 4-pretreated rat. SFPE 4 was infused into a femoral vein with a rate of 10 ㎎/㎏/30 min. Arterial blood pressure was expressed in mmHg. The chart speed was 10 ㎜/min.

IV. DISCUSSION

A cardiovascular disease is a serious problem, today. So, Blood clot and fibrinolytic agent which liquefy blood clot are raising interest. Because the existing fibrinolytic agent is expensive and dangerous, new one is necessary for cardiovascular disease. Among them, there is an attempt of finding fibrinolytic substances from a natural substance. Although the active ingredient in food is small, continuous supply of fibrinolytic substances makes great effect. Pine needles have been used for edibility and medicine since ago.

This article describes the purification and characterization of SFPEs which was isolated from SFPEs, a traditional fermented food. In the course of *in vitro* fibrinolytic activity screening on various extracts from SFPEs. This is the first report in the presence of protocatechuic acid from SFPE 4. When testing all the isolated compound 1 for fibrinolytic activity in vitro assay, the result showed these fibrinolysis possessing a significantly potential activity. Prominently, from the F6.1, compound **1** protocatechuic acid, was the most major compound of the SFPE 4, also revealed a high activity on fibrinolytic activity. Protocatechuic acid from separation process of *Acanthopanacis Cortex* showed good at platelet anti-aggregating substance (Choi *et al.*, 1987). Remarkably, among the isolates, compound **2** shikimic acid, the most major compound of the SFPE 10, also exhibited a significantly fibrinolytic activity. And, from the FB1, compound **2** shikimic acid, was revealed a high activity on fibrinolytic activity. Shikimic acid can be considered as promoting class of

fibrinolysis. Shikimic acid is a hydroaromatic intermediate in the biosynthetic pathways of aromatic amino acid. Owing to its highly functionalized chiral characteristics, shikimic acid has been recognized as an essential starting material for synthesizing neuraminidase inhibitors (marketed as Tamiflu), which are effective in the treatment of influenza (Kim *et al.*, 1997; De Cleraq, 2002). Protocatechuic acid and shikimic acid which are fibrinolytic agent are similar to aspirin used in clinical demonstration. But, if aspirin is used excessively, that is bad for anti-platelet activity and DIC. Therefore, futher investigation and optimization on chemical as well as confirmation of fibrinolytic activity effects of these compounds in cell.

Also, identify and characterize microbial strains from SFPEs, we performed microbial strains screening. 8 different microbial strains Isolates from SFPEs samples showed that No. 2 sample was strong fibrinolytic activity. The No. 2 isolate showed high identity to that of *Acetobacter tropicalis* JY-2. Members of the genus *Acetobacter* from traditional fermented foods are important among the microorganisms (Kim and Choi, 2006). The study could be under way in future to purification and characterization of a fibrinolytic enzyme of *Acetobacter tropicalis* JY-2 in SFPEs.

To know how much useful in cardiovascular disease, in vivo, a rat model, was tested by SFPE 4 and F-SFPE 4.

In blood pressure lowering test using CA secretion detection in white mouse, the CA secretion was inhibited by treatment EtOAc fraction. However, H_2O and BuOH fraction displayed no effect. These results obtained from the present study suggest that EF-SFPE 4 can inhibit the CA secretion evoked by

cholinergic stimulation (both nicotininc and muscarinic receptors) and membrane depolarization from the rat adrenal medulla.

In general, the adrenal medulla has been employed as a model system to study numerous cellular functions involving not only noradrenergic nerve cells but also neurons. During neurogenic stimulation of the adrenal medulla, ACh is released from splanchnic nerve endings and activated cholinergic receptors on the chromaffin cell membrane (Viveros, 1975). This activation initiates a series of events known as stimulus-secretion coupling, culminating in the exocytotic release of CA and other components of the secretory vesicles into the extracellular space. Upon excitation of splanchnic nerves, ACh is released from the nerve terminals, and then activates nicotinic secretion of CA. Based on this fact the present findings suggest that EF-SFPE 4 inhibits the CA secretory responses evoked by nicotinic receptor stimulation as well as by membraned epolarization in the rat adrenal medulla. On the other hand, it is well known that potassium chloride (KCl) opens voltage-dependent ullcium channels by depolarizing the cell membrane of vascular smooth muscle, resulting in increased ir lux of extracellullr Ca^{2+} (Bolton, 1979; Schwartz & Tahaa, 1983; Dube *et al.,* 1985; 1988). In this study, EF-SFPE 4 inhibited the secretory responses of CAs evoked by hlah K. And, EF-SFPE 4 also tDub-dependently depressed the CA secretory response evoked by Bay-K-8644, which is known to activate L-type voltage-dependent Ca^{2+}channels (Garcia et al, 1984; Schramin et al, 1983). This result indicates that EF-SFPE 4 may inhibit Ca 2+ir lux to the rat adrenomedullary cells. As shown in Fla 34, taken together, these experimental results suggest that

EF-SFPE 4 at low concentrations inhibits the CA secretion evoked by cholinergic stimulation (both nicotininc and muscarinic receptors) as well as by membrane depolarization from the rat adrenal medulla.

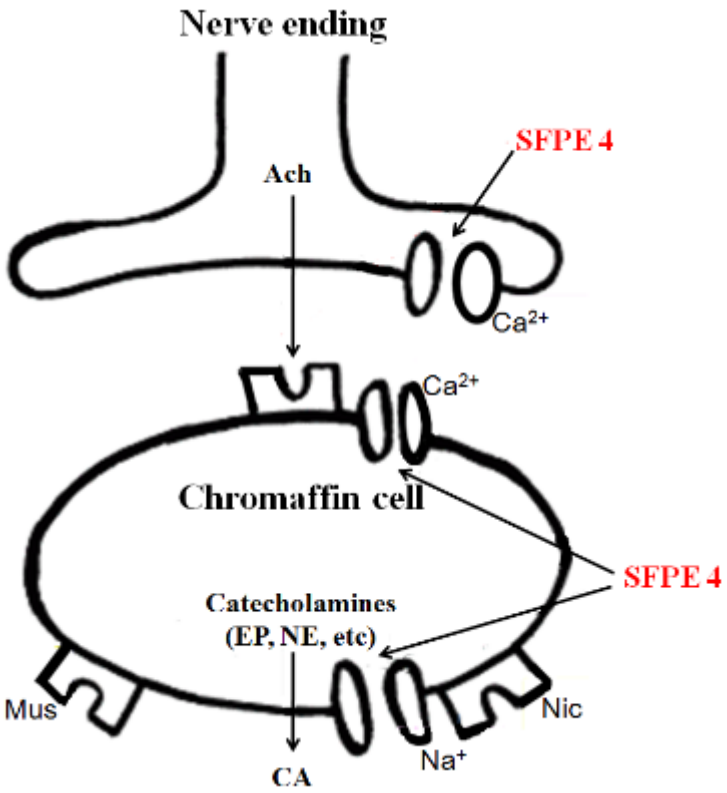


Fig 34. Schematic diagram of possible action site of EF-SFPE 4 at the cholinergic nerve ending-chromaffin cell synapse in the the rat adrenal gland.

SFPE 4 and EF-SFPE 4 was experimented that influence on contractile responses of aortic strips isolated from rats. In the present study, SFPE 4 and EF-SFPE 4 elicited a concentration-dependent inhibition in phenylehrine-induced contractile responses of aortic strips isolated from rats with functional endothelium. Kim and his colleagues (1989) have shown that the contractile responses of vascular smooth muscle induced by $CaCl_2$ and KCl 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, SFPE 4 and EF-SFPE 4 inhibited the high potassium-induced pressor responses as well as phenylephrine-evoked contractile responses in aortic strips isolated from rats. These results suggest that SFPE 4 and EF-SFPE 4 may elicit the antagonistic activity of adrenergic α_1-receptors.

Tannins contained in green tea are also found to induce the depressor effect in rat with renal hypertension (Yokozawa *et al.*, 1994). Extracts of tea (Fitzpatrick *et al.*, 1995) and flavonoids found in tea (Fitzpatrick *et al.*, 1993) have been shown to give vasodilator effects. It was tried to test the effect of intravenous SFPE 4 and EF-SFPE 4 on NE-evoked pressor responses. NE injected intravenously at doses of 1.0, 3.0 and 10.0 ㎍/㎏ caused dose-dependent pressor responses. Therefore, such result suggested and there being a possibility of getting up a pine leaf taking effect SFPE long-term period leisure blood pressure lowering. Also, SFPE 4 (0.3, 1.0 and 3.0 ㎍/㎏/30 min, respectively) dose-dependently suppressed intravenous

NE-induced vasopressor responses in anesthetized SHRs. Based on these findings, the results that NE-induced hypertensive response were markedly depressed by SFPE 4 and EF-SFPE 4, it is that the SFPE 4 and EF-SFPE 4 may be mediated through the blood pressure lowering.

The study could be under way in future to see mechanisms of action of SFPE 4 in decreasing blood pressure and to know blood vessel laxity and CA secretion control in adrenal medulla. From this day forward, we would like to separate the adrenal medulla of material decreasing blood pressure from SPFEs.

V. REFERENCE

Ahn, M. Y., Hahn, B. S., Ryu, K. S., Kim, J, W., Kim, I., and Kim, Y. S. (2003) Purification and characterization of a serine protease with fibrinolytic activity from the dung beetles, *Catharsius molossus*. *Thromb. Res*. 112: 339-347

Anton, A. H., and D. F. Sayre (1962) A study of the factors affecting the aluminum oxidetrihydroxyindole procedure for the analysis of catecolamines. *J. Pharmacol. Exp. Ther*. 138: 360-375.

Astrup, T., and S. Mullertz (1952) The fibrin plate method for estimating of fibrinolytic activity. *Arch. Biochem. Biophys.* 40: 346-351.

Bolton, T. M. (1979) Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev*. 3: 606-718.

Cheong, H., Dilli, P. P, Jun, J. Y., Yeum, C. H., Yoon, P. J., Park, C. G., Kim, M. Y., So, I., Kim, K. W., and Choi, S. (2005) Effects of pine needle extract on pacemaker currents in interstitial cells of Cajal from the murine small intestine. *Mol. Cells*. 20(2): 235-240.

Choi, N. S., Yoo, K. H., Hahm, J. H., Yoon, K. S., Chang, K. T., Hyun, B. H., Maeng, P. J., and Kim, S. H. (2005) Purification and characterization of a new peptidase, bacillopeptidase DJ-2, having fibrinolytic activity: Produced by *Bacillus* sp. DJ-2 from Doen-Jang. *J. Microbiol. Biotechnol*. 15: 72- 79.

Choi, Y., Kim, H. S., and Lee, J. R. (1987) Potential Inhibitors of Platelet Aggregation from Plant Sources, III *J. Nat. Prod*. 50(6): 1059-1064.

Collen, D. (1980) On the regulation and control of fibrinolysis. *Thromb. Haemost.* 43: 77-89.

Collen, D., and H. R. Lijnen (1991) Basic and clinical aspects of fibriof fibrand thrombosis. *Blood*. 78: 3114-3124.

De Clercq, E. (2002) Strategies in the design of antiviral drugs. *Nat. Rev. Drug Discov*. 1: 13- 25.

De-Simone, S. G., C. Correa-Netto, O. A. C. Antunes, R. B. De-Alencastro, and F. P. Silva (2005) Biochemical and molecular modeling analysis of the ability of two p-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms. *J Chromatogr. B Analyt. Technol. Biomed. Life Sci*. 822: 1-9.

Dube, G. P., Y. H. Baik, and A. Schwartz (1985) Effects of novel calcium channel agonist dihydropyridine analogue, Bay K 9644, on pig coronary artery: Biphasic mechanical response and paradoxical potentiation of contraction by diltiazem and nimodipine. *J. Cardiovasc. Pharmacol*. 7: 377-389.

Dube, G. P., Y. H. Baik, C. Van Breemen, and A. Schwartz (1988) Effects of isosorbide dinitrate and diltiazem on Ca^{2+} flux and contraction in artery. *European J. Pharmacol*. 145: 39-47.

Fitzpatrick, D. E., S. L. Hirschfield, and R. G. Coffey (1993) Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am. J. Physiol*. 265: 77-78.

Fitzpatrick, D. E., S. L. Hirschfield, T. Ricci, P. Jantzen, and R. G. Coffey (1995) Endothelium-dependent vasorelaxation caused by various plant extracts. *J. Cardiovasc. Pharmacol*. 26: 90-95.

Formica, J. V., and W. Regelson (1995) Review of the biology of quercetin and related bioflavonoids. *Food Chem. Toxicol*. 33: 1061-1080.

Garcia, A. G., F. Sala, J. A. Reig, S. Viniegra, J. Frias, R. Fonteriz, and L. Gandia (1984) Dihydropyridine Bay-K-8644 activates chromaffin cell calcium channels. *Nature,* 309: 69-71.

Hwang, K. J., Choi, K. H., Kim, M. J., Park, C. S., and Cha, J. H. (2007) Purification and Characterization of a New Fibrinolytic Enzyme of *Bacillus licheniformis* KJ-31, isolated from Korean Traditional Jeot-gal. *J. Microbiol. Biotechnol*. 17(9): 1469-1476

Ichikawa, S., H. Takigawa, and S. Nara (1998) Effects of shoju-sen, a herbal medicine, on unidentified clinical syndrome. *J. Clin. Pharmacol*. New Drugs 47:879- 8887.

Jeong, Y. K., Park, J. U., Baek, H., Park, S. H., and Kong, I. S. (2001) Purification and biochemical characterization of a fibrinolytic enzyme from *Bacillus subtilis* BK-17. *J. Microbiol. Biotechnol*. 17: 89-92.

Jia, Y. H., Jin, Y., Lu, Q. M., and Li, D. S. (2003) Jerdonase, a novel serine protease with kinin-releasing and fibrinogenolytic activity from *Trimeresurus jerdonii venom*. *Acta Biochim Biophys. Sin*. 35: 689-694.

Kee, Y. W., and Lim, D.Y. (2007) Influence of polyphenolic compounds isolated from *Rubus coreanum* on catecholamine release in the rat adrenal medulla. *Arch. Pharm. Res.* 30(10): 1240-1251.

Kim, C. U., W. Lew, M. A. Williams, H. Liu, L. Zhang, S. Swaminathan, N. Bischofberger, M. S. Chen, D. B. Mendel, C. Y. Tai, W. G. Laver, and R. C. Stevens (1997) Influenza Neuraminidase Inhibitors Possessing a Novel Hydrophobic Interaction in the Enzyme Active Site: Design, Synthesis, and Structural Analysis of Carbocyclic Sialic Acid Analogues with Potent Anti-Influenza Activity. *J. Am. Chem. Soc.* 119: 681-690.

Kim, J. M., Park, K. O., and Baik, Y. H. (1989) Effects of antiepileptic drugs on contractile responses of vascular smooth muscles. *Chonnam J. Med. Sci.,* 2(1): 50-59.

Kim, M. H., and Choi, U. K. (2006) Acetic Acid Fermentation by *Acetobacter* sp. SK-7 using Maesil Juice. *J. Food culture*. 21(4): 420 - 425.

Kim, S. B., Lee, D. W., Cheigh, C. I., Choe, E. A., Lee, S. J., Hong, Y. H., Choi, H. J., and Pyun, Y. R. (2006) Purification and characterization of a fibrinolytic subtilisin-like protease of *Bacillus subtilis* TP-6 from an Indonesian fermented soybean, Tempeh. *J. Ind. Microbiol. Biotechnol*. 33: 436-444.

Lee, H. J., Cui, C. B., Choi, H. T., Kim, S. H., Ham, Y. A., Lee, D. S., and Ham, S. S. (2005) Quality Characteristics of the Vaporizeharaquid of Water-boiled Pine Needle, *Korean J. Food Preserv*. 2: 107-111.

Lee, J. S., Baik, H. S., and Park, S. S. (2006) Purification and characterization of two novel fibrinolytic proteases from mushroom, *Fomitella fraxinea*. *J. Microbiol. Biotechnol.* 16(2): 264-271.

Lim, D. Y., Kim, C. D., and Ahn, K. W. (1992) Influence of TMB-8 on secretion of catecholamines from the perfused rat adrenal glands. *Arch. Pharm. Res*. 15(2): 115-125.

Lim, D.Y. (2008) PCRC inhibits catecholamine secretion from adrenal medulla of SHRs. *The 31stAnnual Scientific Meeting of JSH 2008 Program and Abstracts*. pp. 424.

Mihara, H., H. Sumi, T. Yoneta, H. Mizumoto, R. Ikeda, and M. Seiki (1991) A nove1 fibrinolytic enzyme extracted from the earthworm. *Lumbricus rubellus. Jpn. J. Physiol*. 41: 461-472.

Peng, Y., X. Yang, and Y. Zhang (2005) Microbial fibrinolytic enzymes: An overview of source, production, properties, and thrombolytic activity *in vivo*. *Appl. Microbiol. Biotechnol*. 69: 126-132.

Schwartz, A., and N. Taira (1983) Calcium channel-blocking drugs: A novel intervention for the treatment of cardiac disease. *Circ. Res. (American Heart association Monograph)*. 52: 1-183.

Sumi, H., N. Nakajima, and H. Mihara (1992) Fibrinolysis relating substances in marine creatures. *Comp. Biochem. Physiol. B*. 102: 163-167.

Sung, K. C. (2004) Characteristics and Analysis of Natural Pine-Needles Extract. *J. Korean Oil Chem. Soc*. 4: 320-326.

Sung, K. C., and Kim, K. J. (2005) Tyrosinase activated inhibition effect and analysis of pine needle extract. *J. Korean Oil Chem. Soc*. 22: 71-76.

Tallarida, R. J., R. B. Murray (1987) *Manual of pharmacologic calculation with computer programs*. 2nd ed., pp 132. Speringer-Verlag, NY, USA.

Viveros, O. H. (1975) Mechanism of secretion of catecholaminies from adrenal medulla. *In handbook of physiology, Endocrinology*. Vol. Sect 7. pp. 389-426. The adrenal gland. American physiological society, Washington DC, USA.

Voet, D. and Voet, J. G. (1990) *Biochemistry*. 2nd ed., pp. 1087-1095. In John Wiley and Sons, New York, USA.

Wada, Y., K. Satoh, and N. Taira (1985a) Cardiovascular profile of Bay-K-8644, a presumed calcium channel activator in the dog. *Naunyn-Schmiedebergs Arch. Pharmacol*. 328: 382-387.

Wada, A., H. Takara, F. Izumi, H. Kobayashi, and N. Yanagihara (1985b) Influx of 22Na through acetylcholine receptor-associated Na channels: relationship between 22Na influx, 45Ca influx and secretion of catecholamines in cultured bovine adrenal medullary cells. *Neuroscience*. 15: 283-292.

Wakade, A. R. (1981) Studies on secretion of catecholamines evoked by acetylcholine or transmural stimulation of the rat adrenal gland. *J. PhysioL*. 313: 463-480.

Wakanyaku Medical Institute (1996) The clinical effects of Shoju-sen. Reports 1 and 2, Wakanyaku Medical Institute, Tokyo (in Japanese).

Yen, G. C., Duh, P. D., Huang, D. W., Hsu, C. L., and Fu, T. U. C. (2008) Protective effect of pine (Pinus morrisonicola Hay.) needle on LDL oxidation and its anti-inflammatory action by modulation of iNOS and COX-2 expression in LPS-stimulated RAW 264.7 macrophages. *Food Chem. Toxicol*. 46(1): 175-185.

Yokozawa, T., H. Oura, S. Sakanaka, S. Ishigaki, and Kim, M. (1994) Depressor effect of tannin in green tea on rats with renal hypertension. *Biosci. Biotech. Biochem.* 58: 855-858.

Zenebe, W., and Pecháň iová, O. (2002) Effect of red wine polyphenolic compounds on the cardiovascular system. *Bratisl. Lek. Listy*. 103: 159-165

저작물 이용 허락서

학 과	생명공학과	학 번	20087064	과 정	석사
성 명	한글: 정 주 영 한문: 鄭 周 永 영문:Juyoung Jung				
주 소	광주광역시 남구 진월동 리채 APT 301 동 301 호				
연락처	E-MAIL : hahaha-83@hanmail.net				
논문제목	한글: 솔잎 발효에 따른 혈전분해 물질 정체 및 생리적 특성 영어: Characterization and purification of fibrinolytic compounds during fermentation of pine needle extract				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부: 동의(○) 반대( )

2009 년 2 월 25 일

저작자: 정 주 영 (서명 또는 인)

조선대학교 총장 귀하