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Development of natural resources for cardiovascular disease improvement from *Pinus densiflora*

조선대학교 대학원

생명과학과

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Development of natural resources for cardiovascular disease improvement from *Pinus densiflora*

A thesis submitted to the Graduate School of the Chosun University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

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ABBREVIATION

ACE	Angiotensin converting enzyme
AT_1 receptor	Angiotensi II receptor, type 1
COX-1	Cyclo-oxygenase-1
CVD	Cardiovascular disesase
DMSO	dimethyl sulfoxide
eNOS	endothelial nitric oxide synthase 3
EtOAc	Ethyl acetate
FBS	Foetal bovine serum
FPE	fermented pine needle extract
HA	Hippuric acid
HHL	Hippuryl-His-Leu

MTT	3-(4,5-dimethyl-2yl)-2,5-dipheyltetrazolum bromide
n-BuOH	*n*-butyl alcohol
NO	Nitric oxide
PBS	phosphate buffered saline
PCA	Protocatehcuic acid
PE	Pine needle extract
RAS	Renin angiotensin system
SA	Shikimic acid
sACE	somatic ACE
SVEC 4-10	SV40 transformed endothelial cell
tACE	testis ACE
tPA	tissue plasminogen activator
TXA2	Throboxane A2

ABSTRACT

Development of natural resources for cardiovascular disease improvement from *Pinus densiflora*

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솔잎은 소나무 과에 속하며 세계적으로 넓게 분포하고 있다. 한국, 중국, 일본 등 동아시아의 각 나라에서는 소나무의 잎, 열매, 껍지, 꽃가루 등 다양한 부위를 식용 또는 건강 증진을 위한 식사 보충제로 사용되고 있다. 건강 증진을 위한 솔잎의 효능은 항염증, 항균, 항바이러스, 항우울제, 항고혈압과 트리글리세리드 감소 효과 등 다양한 생리학적 및 약리학적 활성이 알려져 있다. 이전 연구에서 솔잎발효액은 fibirnolysis 및 antihypertension activity에 대해 연구되어졌다. 솔잎발효액에서 fibrinolysis 물질인 protocatehcuic acid과 shikimic acid은 에틸아세테이트와 부탄올 층에서 각각 분리되었다. protocatehcuic acid과 shikimic acid은 낮은 pH 범위에서(2 - 4) thrombus 분해가 완벽하게 발생되었다. fibrin 형성 억제 활성은 UV spectrophotometer를 이용하여 fibrin solution의 탁도 변화를 통해

확인하였고, protocatehcuic acid과 shikimic acid은 fibrin solution의 탁도가 증가되는 것을 억제하였고, 이는 protocatehcuic acid과 shikimic acid이 혈전 형성을 억제한다는 것이 확인되었다. Protocatehcuic acid과 shikimic acid의 혈전분해 방식을 확인하기 위해 SDS-PAGE를 이용하여 분석하였고, 분석결과 protocatehcuic acid과 shikimic acid의 fibrinolysis 방법은 plasmin과는 다르게 분해한다는 것을 확인하였다. Plasmin은 fibrin의 Aα, Bβ 단백질 밴드가 사라졌지만, protocatehcuic acid과 shikimic acid은 피브린의 Aα, Bβ, γ 단백질 밴드가 유지되었다. protocatehcuic acid과 shikimic acid의 양적 변화를 솔잎발효액에서 측정하였다. 발효과정 동안 protocatehcuic acid는 지속적으로 증가하였지만, shikimic acid의 양은 특별한 변화가 없었다. 솔잎발효액은 protocatehcuic acid과 shikimic acid의 우수한 공급원이며 필요한 충분한 양을 값싸고 충분한 양을 공급할 수 있다. Renin angiotensin system에서 angiotensin converting enzyme은 angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile)을 angiotensin II(Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile)로 바꾸는 중요한 단백질이다. Angiotensin II은 angitensin II receptor와 결합하여 aldosterone을 생산하고 Na^+를 흡수하여 혈압을 상승시킨다. 적송의 잎에서 anhypertension 물질을 연구하기 위해서, 적송의 잎을 물, 에틸아세테이트, 부탄올 층으로 분리하여 angiotensin converting enzyme 억제 활성을 분석하였다. 가장 잠재적으로 angiotensin converting enzyme 억제 활성을 가지고 있는 것은 에틸아세테이트 층(5 mg/mL)에서 확인 되었다. 컬럼 크로마토그래피로 angiotensin converting enzyme 억제 물질의 정제 후, antihypertension activity은 역상 액체크로마토그래프를 이용하여 측정하였다. 두 개의 활성 물질을 분리 및 정제하였고, 구조분석 결과

angiotensin converting enzyme 억제 물질은 각각 abieta-8,11,13-trien-18-oic acid (dehydroabietic acid)와 8(17),12,14-Labdatriene-19-oic acid (communic acid)으로 확인되었다. Antihypertension 활성의 분석을 위해, 역상 액체크로마토그래프를 이용하여 hippuric acid의 양을 측정하였다. Abieta-8,11,13-trien-18-oic (3 mM)과 8(17),12,14-Labdatriene-19-oic acid (3 mM)은 N-Hippurly-His-Leu에서 hippuric acid 생산을 억제하였다. 또한, abieta-8,11,13-trien-18-oic acid와 8(17),12,14-Labdatriene-19-oic acid은 endothelial cell에서 angiotensin converting enzyme와 angiotensin II receptor 발현을 조절하였다. 적송의 잎에서 분리된 두 개의 abieta-8,11,13-trien-18-oic acid (dehydroabietic acid)과 8(17),12,14-Labdatriene-19-oic acid (communic acid)는 새로운 angiotensin convering enzyme inhibitor임을 확인하였다.

ABSTRACT

Development of natural resources for cardiovascular disease improvement from *Pinus densiflora*

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Pine trees (Pinus densiflora Sieb. et Zucc) belong to the family Pinaceae and are widely distributed around the world. In East-Asian countries such as Korea and China, Japan, various parts of pine trees, including the needles, cones, cortices, and pollen, are widely consumed as foods or dietary supplements to promote health and have shown a wide spectrum of biological and pharmacological actions such as anti-inflammatory, antibacterial, antiviral, antidepressant, antihyperternsion, and triglyceride decreasing effects. We have previously reported that the fermented pine needle extract (FPE) from these plants possesses fibrinolytic activity and antihypertension. Two fibrinolytic compounds, protocatechuic (PCA) and shikimic acids (SA), were isolated from the ethyl acetate- and n-butyl alcohol-soluble fractions of FPE, respectively. Fibrin clots lysis in compounds PCA and SA occurred completely in the pH 2-4 range.

These compounds strongly suppressed fibrin clots formation, which was determined by measuring turbidity. The SDS-PAGE pattern demonstrated that plasmin fibrinolysis was different from that of chemicals PCA and SA. Plasmin disassembles the Aα and Bβ chains of fibrin, but chemicals PCA and SA maintained the Aα, Bβ, and γ fibrin chains after fibrinolysis. Furthermore, we measured the changes in the quantities of compounds PCA and SA that were obtained from FPE. While the fermentation progressed, we determined that the quantity of compound PCA steadily increased, while the quantity of SA did not demonstrate significant changes. FPE is an excellent resource for chemicals PCA and SA and can provide inexpensive and sufficient quantities for industrial-scale extraction. The Angiotensin converting enzyme (ACE) is the key enzyme catalyzes angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile) to angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile) in renin-angiotensin system. Angiotensin I produces aldosterone in combination with angiotensin II receptor, which increases blood pressure through absorption of Na^+. To investigate the antihypertensive compounds from Pinus densiflora Sieb. et Zucc (red pine) needles, water, ethyl acetate - and n-butyl alcohol-soluble fractions from red pine needles were screened for the inhibitory activity against ACE. The most potent ACE inhibitory activity was detected in the ethyl acetate alcohol-soluble fractions (5 mg/mL). After the purification of ACE inhibitor compounds with column chromatography, antihypertensive activity was determined by measuring Revers Phase-HPLC. We obtained two active compounds, abieta-8,11,13-trien-18-oic and 8(17),12,14-Labdatriene-19-oic acids. To measure

quantitative analysis of antihypertensive activity, we analyzed the quantities of hippuric acid using RP-HPLC. abieta-8,11,13-trien-18-oic acid (3 mM) and 8(17),12,14-Labdatriene-19-oic acid (3 mM) inhibited production hippuric acid in N-Hippuryl-His-Leu. Also, abieta-8,11,13-trien-18-oic acid (dehydroabietic acid) and 8(17),12,14-Labdatriene-19-oic acid (commnunic acid) regulated ACE and angiotensin II receptor expression in endothelial cells. We determined that abieta-8,11,13-trien-18-oic and 8(17),12,14-Labdatriene-19-oic acids separated from the red pine needles are new ACE inhibitors.

I. Introduction

Pinus densiflora (red pine) is widely distributed around East-Asian countries including Korea, Japan, China, and southeastern Russia. Although red pine needles contain growth inhibitory substances and possess allelopathic potential in herbaceous plants, various parts of red pine are used as foods or dietary supplements to promote health (Cabon, 2006; Kim et al., 2000). Pine needle extract (PE) was reportedly identified to have biological and pharmacological activities, including anti-oxidant, anti-inflammatory, anti-bacterial, anti-cholesterol, gastrointestinal motility control, and fibrinolysis effects (Choi et al., 2007; Jun et al., 2003; Park et al., 2008a; Park et al., 2008b). In particular, we have discovered fibrinolysis effect of PE for the first time (Park et al., 2008a; Park et al., 2008b).

Cardiovascular disease (CVD) is responsible for one-third of global deaths and is a leading and increasing contributor to the global disease burden (Diane et al., 2002). Importantly, CVD is eminently preventable. In order to achieve significant reductions in the avoidable CVD burden, a combination of population-based and high-risk strategies necessary. These strategies should target lifestyle-related risk factors such as unhelathy diet, physical inactivity and tobacco use, as well as the intermdiate mainfestations of these lifestyles; hypertension, glucose intolerance, and hyperlipidemia. In addition, strategies aimed at improving management of those already affected by CVD should be an integral component of a comprehensive approach for the prevention and control of CVD.

Both coagulation and fibrinolysis are precisely regulated by the fine-tuned participation of substrates, activators, inhibitors, cofactors and receptors. Blood clot formation occurs when fibrinogen is converted by thrombin to fibrin, which is the structural protein that assembles into the fibrin polymer (Cesarman et al., 2005).

Thrombin itself forms after prothrombin is proteolytically cleaved by Factor Xa. Fibrin is a major protein component of blood clots. Human fibrinogen is a plasma glycoprotein that participates in the final phase of blood coagulation. It is composed of two sets of three polypeptides designated Aα, Bβ and γ with molecular masses of 66, 52, and 46 kDa, respectively (Mckee et al., 1996; Williams et al., 1998), which combine to a 340-kDa bilaterally symmetrical glycoprotein consisting of six polypeptides (AαBβγ)2 that are held together by 29 disulfide bonds (Henschen et al., 1986).

Fibrinolysis is mediated by plasmin, which is a serine protease that cleaves the fibrin network.5,6 Plasmin is derived from plasminogen by tissue plasminogen activator (tPA) or urokinase-type plasminogen activator5,7 in the presence of fibrin, which accelerates the proteolysis rate (Lord S. T., 2011; Zamolodchikov et al., 2012) (Fig 1).

Over the last decade, potent fibrinolytic components have been discovered from various sources such as earthworms (Mihara et al., 1990), snake venoms (Jia et al., 2003), insects (Ahn et al., 2003), mushrooms (Lee et al., 2006), and fermented food products such as Japanese natto (Sumi et al., 1992) and Korean

chungkook-jang soy sauce (Jeong et al., 2001).

Aspirin, a normally analgesic drug, is among the most well-known synthetic antithrombotic reagents. The antithrombotic effects of aspirin reflect its ability to inhibit thromboxane A2 (TXA2) synthesis, which is a potent inducer of platelet aggregation and vasoconstriction, by inactivating platelet cyclo-oxygenase-1 (COX-1) (Patrono et al., 2005).

Additionally, fibrinogen is reportedly acetylated by aspirin, which alters fibrin (Bjornsson et al., 1989). Aspirin-mediated fibrinogen acetylation affects the fibrin network structure by making the network more porous (Antovic et al., 2005; Fatah et al., 1996; Williams et al., 1998). A more porous fibrin network has been associated with facilitated fibrinolysis, which may explain part of aspirin's antithrombotic effects (Ajjan et al., 2009; Collet et al., 2006).

Protocatechuic acid (PCA) and shikimic acid (SA) are secondary metabolites that have been found only in microorganisms and plants through the shikimate pathway (Herrmann et al., 1995). PCA and SA were simple phenolic compounds that are widely distributed in nature, and display many useful biological activities. PCA could protect against epithelial malignancy in different tissues as well as cardiovascular diseases and may act as a tumor modulator to reduce cancer risk (Lin et al., 2007; Tanaka et al., 2011). PCA reportedly inhibited lipopolysaccharide-induced liver damage in rats and protected against H_2O_2-induced apoptosis in PC12 cells (Chou et al., 2010; Lin et al., 2003). It was also shown that PCA inhibited tyrosinase activity, melanin formation, promoted cell proliferation and neural stem cell apoptosis (Shui et al., 2006; Shui

et al., 2009). SA was an essential compound in plant aromatic amino acids biosynthesis pathways and was a major precursor for many plant alkaloids (Hiroki et al., 2009). In plants and microorganisms, SA can lead to the L-phenylalanine, L-tyrosine and L-tryptophan biosynthesis (Enrich et al., 2008). SA has a variety of biological activities; it was reportedly a potent competitive inhibitor of C4 phosphoenolpyruvate carboxylase (Colombo et al., 1998), and also modulated innate immunity when combined with quercitin (Bertelli et al., 2008). In addition to SA, the derivatives of SA were used as herbicides in agriculture and horticulture industry without any negative effect on mammals (Jiang et al., 1999). Additional useful utility of SA was a synthetic building block for the production of oseltamivir (Tamiflu).

Hypertension is already a highly prevalent risk factor for CVD throughout the industrialized world. It is becoming an increasingly common helath problem worldwide because of increasing longevity and prevalence of contributing factors such as obesity, physical inactivity and an unhealthy diet (Wada et al., 1985; Feio et al., 1997). The current prevalence in many developing countries, particularly in urban societies, is already as high as those seen in developed countries (Tolmacheva et al., 2006; Jayaram et al., 1994).

Hypertension plays a major etiologic role in the development of cerebrovascular disease, ischemic heart disease, cardiac and renal failure. Treating hypertension has been associated with about a 40% reduction in the risk of stroke and about a 15% reduction in the risk of myocardial infarction (Kinouchi et al., 2000). Although the treatment of hypertension has been shown

to prevent CVD and to extend and enhance life, hypertension remains inadequately managed everywhere (Prinz et al., 2002; Vidotti et al., 2004; Yoon et al., 2000; Bernsterin et al., 1989; Acharya et al., 2003; Ehlers et al., 1989; Unger, 2002). In addition, hypertension often coexists with other cardiovascular risk factors, such as tobacco use, diabetes, hyperlipidemia and obesity, which compound the cardovascular risk attributable to hypertension (Prinz et al., 2002; Vidotti et al., 2004; Yoon et al., 2000).

The renin angiotensin system (RAS) play a important role in the blood pressure regulation. Major compounds are angiotensinogen, angiotensin converting enzyme, angiotensin I and II, angiotensin II receptor type I (Fig. 2). ACE (EC 3.4.15.1) belongs to the zinc protease and is located in the endothelial cell. ACE function is to cleave his-leu from angiotensin I to form angiotensin II ACE has N-terminal and C-terminal and the active sites interact to different substrates ACE inhibition considered an important treatment of high blood pressure (Actis-Goretta et al., 2003).

The first orally active inhibitor is captopril, and it was made based on known inhibitors of another carboxy-peptidase A. Captorpril, ACE inhibitor, competitively intercept the activity of ACE, thereby decreasing circulation and local concentraion of angiotensin II. Decreasing angiotensin II is reducing aldosterone and vasopressin (Atlas, 2007). Angiotensin II is a important effector of vasoconstriction, cell growth, sodium and water retention. Angiotensin II appears to promote endothelial a functional disorde, inflammation, oxidative stress, insulin resistance, and reduced β-cell hyper-response. The connection between the RAS and hypertension has led to compelling indications to block the formation or activity of angiotensin II through use of ACE inhibitiors and angiotensin receptor blocker (Ferrario, 2010)

Diterpene resin acids are an important compound to prevent herbivores and pathogens (Diane et al., 2002). The biological activity of diterpene resin acids,

such as antimicrobial, antiulcer, and cardiovascular activityes is the most represented for this class of diterpenoids. Dehydroabietic acid and its derivatives exhibit antiulcer antiulcer (Wada et al., 1985), antimicrobial (Savluchinske et al., 1997), anxiolytic (Tolmacheva et al., 2006), antiviral (Tagat et al., 1994), antitumor (Prinz et al., 2002), and cytotoxic activities (Kinouchi et al., 2000).

In this study as a continuation of our search for active fibrinolytic and antihypertension compounds from FPE and needle of red pine, it was found for that fibrinolysis and antihypertension compounds in FPE and needle of red pine, respectively. Herein, we report as potential novel fibrinolytic agents and antihypertension compounds. To study interaction of ACE and ACE inhbitory, also, construction of the ACE gene was transformed using pET-15b with the cDNA of the mouse.

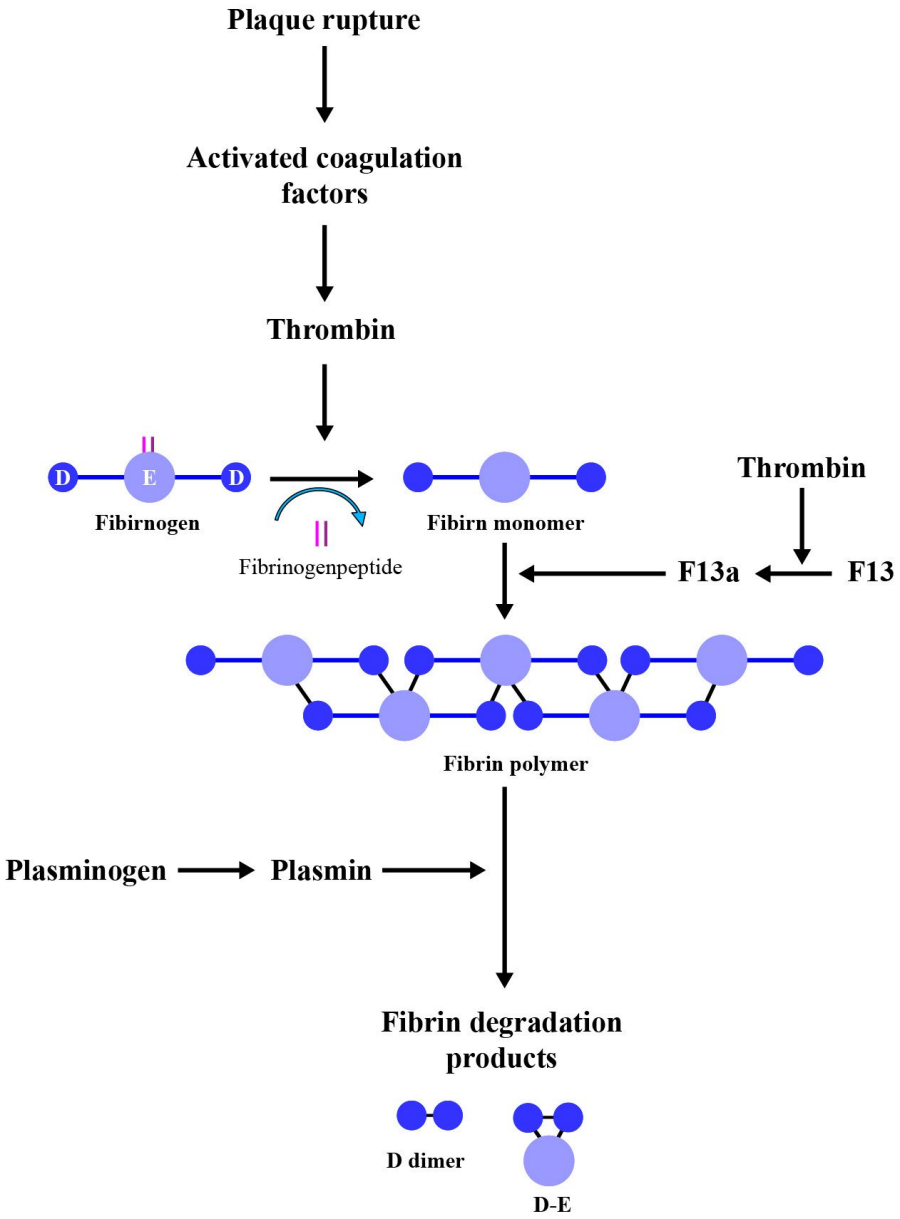

Figure 1. The overview of fibrin clot formation and fibrinolysis.

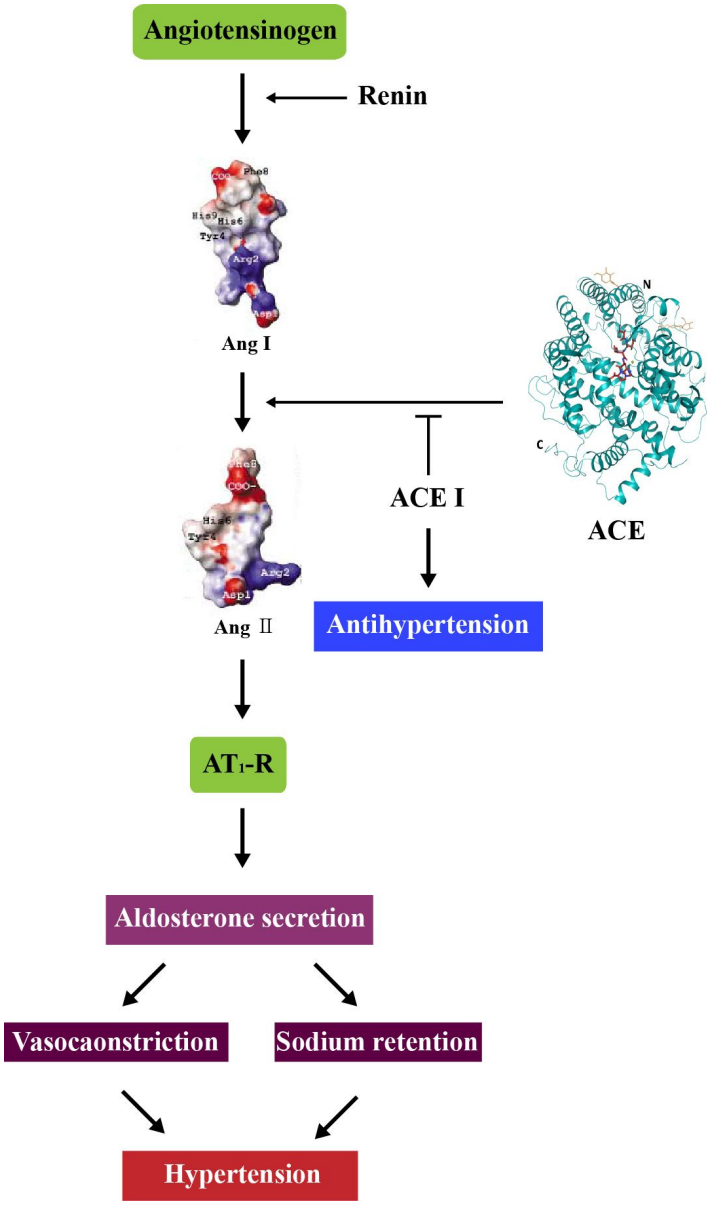

Figure 2. The classical renin angiotensin converting enzyme pathway. ACE; angiotensin converting enzyme, ACE I; angiotensin converting enzyme inhibitor, Ang I; angiotensin I, Ang II; angiotensin II, AT_1-R; angiotensin II receptor (type I).

Capter 1.

Screening of compounds with biological activity on fibrinolysis from fermented *Pinus densiflora* extract

1.1 Introduction

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1999). Additional useful utility of SA was a synthetic building block for the production of oseltamivir (Tamiflu).

1.2 Material and Method

1.2.1 General experimental procedures

UV spectra were recorded in MeOH on a UV-vis spectrophotometer (Optizen, 3220UV). NMR spectra were attained using a Varian Unity-INOVA spectrometer that was operated at 500 MHz for 1H NMR and at 125 MHz for 13C NMR with complete proton decoupling at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). The deuterated solvent for NMR analysis (CDCl3) was purchased from CIL (Cambridge Isotope Laboratory, USA). The MS data were obtained using a Micro mass QTOF2 (Micro mass, Wythenshawe, UK) mass spectrometer. Silica gel (Merck, 63 um ~ 200 um particle size) and C-18 silica gel (Merck, 75 um particle size) were used for column chromatography. TLC was obtained using silica gel 60 F254 and RP-18 F254 plates that were purchased from Merck. To isolate compounds 1 and 2, HPLC was performed using a Gilson (Middleton, USA) system with a UV detector and an ODS-H80 column (20 × 150 mm, 4 μm particle size). To quantify the two compounds, a reversed-phase HPLC with a pack polymer C18 column (6 μm particle size, 4.6 mm, 250 mm) from the YMC Corporation, and a Shimadzu HPLC system (Tokyo, Japan) consisting of a LC-20AT pump, CTO-10AS column oven, and an SPD-20A UV detector was employed. To measure turbidity, an Eon UV spectrophotometer from BioTek Instruments, Inc. (Winooski, USA) was used.

Gen 5.2 software was used for the turbidity analysis. Fibrinogen, thrombin, protocatechuic acid and shikimic acid were purchased from the Sigma Aldrich Co. (St. Louis, USA). Compounds for the assay were dissolved at a concentration of 5 mM in distilled water stock solution. The stock solution was diluted to the desired final concentrations with fibrin and fibrinogen solution just before use. All of the solvents used for extraction and isolation were of analytical grade.

1.2.2 Plant material

Fresh red pine needles (Pinus densiflora Sieb. et Zucc.) were picked up from red pine in the Gokseong province, Jeollanam-Do, Southern Korea. Harvested red pine needles were cleaned with tap water (cleaned with 5% charcoal) and dehydrated with the spin-drier. The dehydrated pine needles were crushed and extracted with the press-juice extractor.

1.2.3 Fermented pine needle extract (FPE) preparation

To recover the supernatant, the pine needle extract (PE) preparation was allowed to settle at 4 ºC for 3 h. FPE were stored at 23±2 ºC for years for fermentation.

1.2.4 Extraction and Isolation

The dried and powdered fermented pine needle extract (*Pinus densiflora* Sieb. et Zucc.) (FPE) (214.6 g) was dissolved in H_2O (1.0 L) and then partitioned (1:1) with *n*-hexane to give an n-hexane-soluble fraction. The H_2O layer was dried and H_2O (500 mL) was then added. The aqueous solution was partitioned with ethyl acetate (EtOAc) to separate the EtOAc-soluble fraction (20.0 g) from the H2O-soluble fractions. The EtOAc-soluble fraction (20.0 g) was passed over a silica gel column and eluted with n-hexane:acetone (6:1 to 0:1) to give nine fractions (F1-F9). The F6 fraction was loaded onto an HPLC ODS-H 80 column to yield protocatechuic acid (1) (35 mg) using a mixed solvent of MeOH-H2O (33:67) as an eluent. After isolating the EtOAc layer, the H_2O layer was dried, and H_2O (500 mL) was then added. This aqueous solution was partitioned with *n*-butyl alcohol (*n*-BuOH) to separate the *n*-BuOH-soluble fraction (250 mg) from the H_2O-soluble fractions. The *n*-BuOH-soluble fraction (250 mg) was passed over a Sephadex LH-20 column and eluted with MeOH to give three fractions (F1-F3). The F1 fraction was applied to an HPLC ODS-H 80 column to yield shikimic acid (90 mg) using a mixed MeCN-H_2O (5:95) solvent as an eluent.

1.2.5 Fibrinolysis activity assay in fibrin clots and fibrinogen solution

To measure the fibrinolysis activity of PCA and SA in fibrin clot solution, fibrinogen (0.45 mg/mL) and thrombin (0.25 U/mL) were incubated at 37 ºC for

2 h. After fibrin clot formation, the fibrin clot solution was incubated with various concentrations of PCA and SA (0, 0.25, 0.5, and 1 mM) at 37 ºC, and the reaction was stopped after 30 min. To identify how varied pH changes the fibrinolysis activity of PCA and SA in test tubes, the fibrin clot solution was incubated with solutions of various pH along with PCA and SA (pH 2-7) at 37 ºC, and the reaction was stopped after 30 min. The fibrin clot solution was also filled in prepared test tubes, which were sealed on one side with parafilm, and incubated at 37 ºC for 2 h. After fibrin clot formation, the fibrin clot solution was incubated with PCA and SA at various pH levels (pH 2-7) at 37 ºC, and the reaction was stopped after 30 min. To compare the fibrinolysis patterns of fibrin and fibrinogen solution that was treated with PCA, SA, and aspirin with plasmin, the fibrin and fibrinogen chain composition was analyzed by 12% SDS-PAGE. To measure fibrin clot formation after treatment with PCA, SA, and aspirin in fibrinogen solution, fibrin clotting was initiated in H_2O (pH 7 containing 100 mM CaCl2) at 37℃ with final fibrinogen concentrations of 0.45 mg/mL. The fibrinogen solution was incubated with PCA, SA, aspirin, and thrombin (0.25 U/mL). The final concentrations of PCA, SA, and aspirin were adjusted to 1 mM, and the reaction was stopped after 30 min. Fibrin clot and fibrinogen solution turbidity measurements were modified as previously described.41 Turbidity was monitored at a wavelength of 350 nm on the Eon multiwall plate reader (Biotek Instruments Inc.).

1.2.6 Quantitative analysis of protocatechuic and shikimic acids

Quantitative analysis of PCA in FPE was performed using HPLC with a pack polymer C18 column (4.6 × 250 mm, 6 μm particle size, Kyoto, Japan). The mobile phase was MeOH in H_2O containing 0.1% formic acid (0−25 min: 30% MeOH, 25−28 min: 30−100% MeOH, 28−38 min: 100% MeOH, 38−42 min: 30-100% MeOH, 42−45 min: 30% MeOH) at a flow rate of 3.0 mL/min and UV detection at 205 and 254 nm to obtain PCA (tR = 17.8 min). Quantitative analysis of SA in FPE was performed using HPLC with a pack polymer C18 column (4.6 × 250 mm, 6 μm particle size, Kyoto, Japan) and a MeCN in H_2O mobile phase containing 0.1% formic acid (0−13 min: 5% MeCN, 16−20 min: 80% MeCN, 21−25 min: 5%) at a flow rate of 3.0 mL/min and UV detection at 205 and 254 nm, tR = 10.0 min.

1.3 Results and discussion

1.3.1 Isolation and purificaiton of fibrinolysis compounds

The fermented pine needle extract (FPE) demonstrated strong fibrinolysis activity.27,28 The FPE with fibrinolysis activity was fractionated with *n*-hexane, ethyl acetate- (EtOAc) and *n*-butyl alcohol (*n*-BuOH). Among these, the EtOAc, and *n*-BuOH-soluble fractions exhibited strong fibrinolysis activity. The EtOAc-soluble fraction that exhibited strong fibrinolysis activity was further purified by repeated silica gel column/HPLC, and the *n*-BuOH soluble fraction was purified by Sephadex LH-20 column/HPLC (Fig. 1). The chemical structures of the protocatechuic acid (PCA) from the EtOAc-soluble fraction and the shikimic acid (SA) from the n-BuOH-soluble fraction of FPE were identified using spectroscopic data (Fig. 2 and 3).

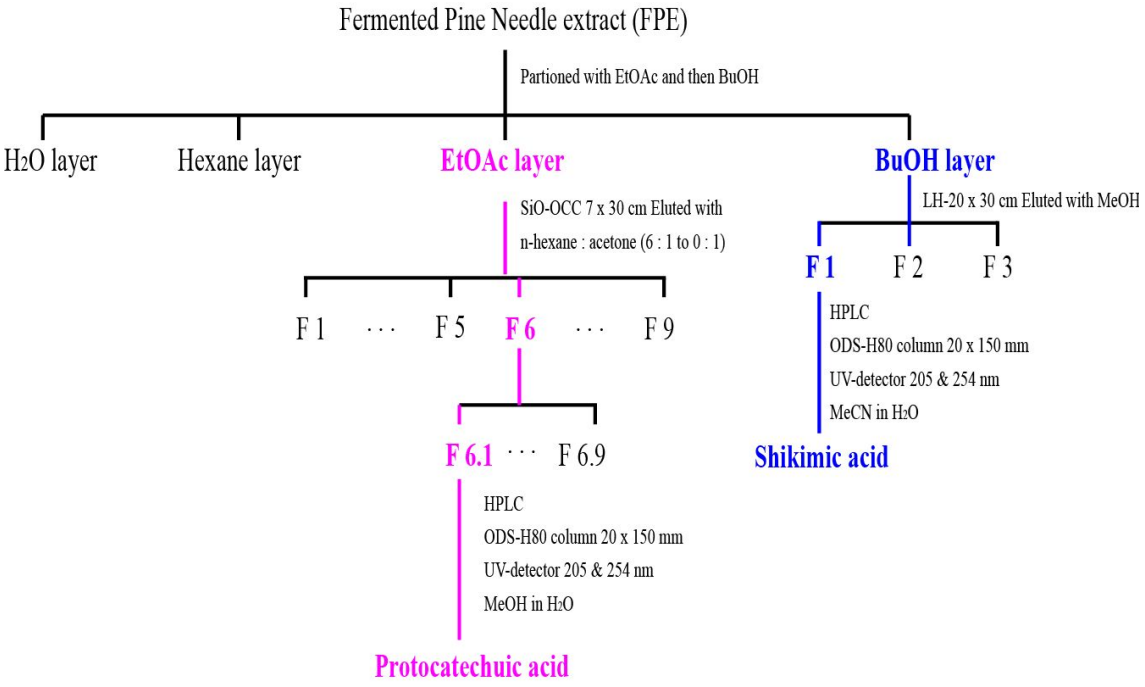

Figure 3. Procedure for islating protocatechuic acid and shikimic acid from fermented pine needle extract (FPE). The ethyl acetate-soluble fraction exhiting strong fibrinolysis activity was further purified by repeated silica gel column and HPLC, and the *n*-butyl alcohol-soluble fraction was purified with a Sephadex LH-20 column and HPLC. The structures of protocatechuic acid from the FPE ethyl acetate-soluble fraction and shikimic acid from the FPE *n*-buthyl alcohol-soluble were identifed by ^{1}H NMR and ^{13}C NMR spectroscopy.

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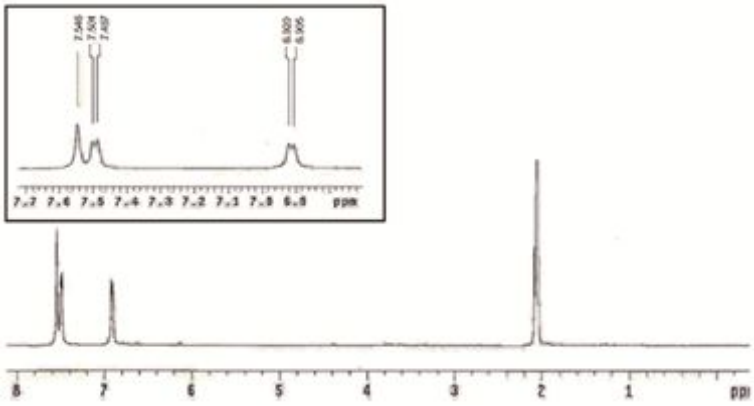

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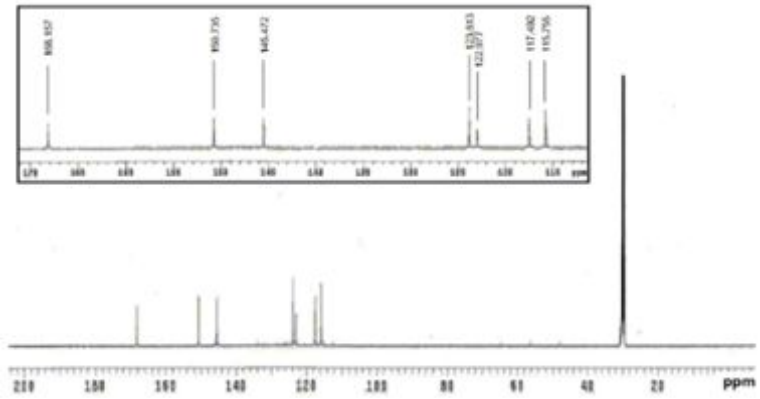
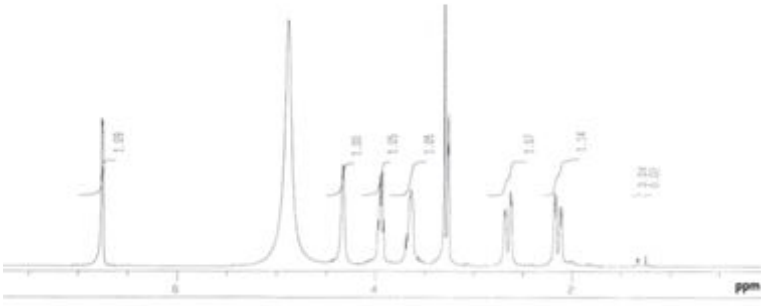


Figure 4. ^{1}H and ^{13}C NMR spectra of protocatechuic acid isolated from fermented pine needle extract. A. ^{1}H NMR spectrum. B. ^{13}C NMR spectrum.

A



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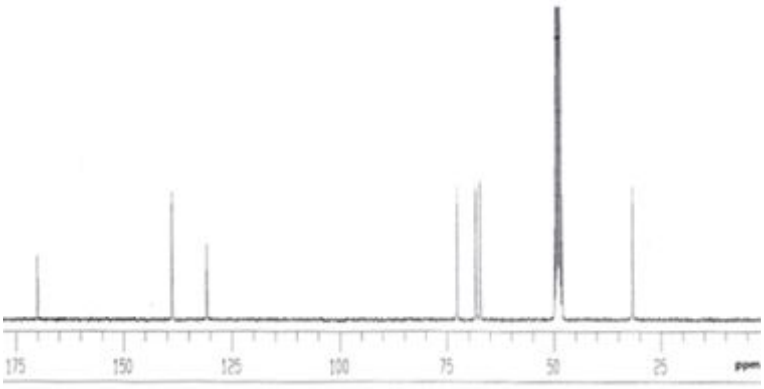

Figure 5. ^{1}H and ^{13}C NMR spectra of shikimic acid isolated from fermented pine needle extract. A. ^{1}H NMR spectrum. B. ^{13}C NMR spectrum.

1.3.2 Fibrinlysis activity of protocatechuic acid and shikimic acid

The fibrinolysis of PCA and SA was determined by measuring turbidity using a UV spectrophotometer. To measure fibrinolysis activity, chemicals PCA and SA were added to the fibrin clot solution. When PCA and SA (1 mM) were added to the fibrin clot solution, the turbidity of fibrin clot solution dramatically decreased during a 30 min incubation, which clearly indicated that the fibrin clot was dissolved.41,42 Also, this activity seems to be dependent upon a certain range of concentrations (0.25 – 1.0 mM) of PCA and SA (Fig. 4). These results suggest that PCA and SA are active molecules possessing potential fibrinolytic activity in the FPE.

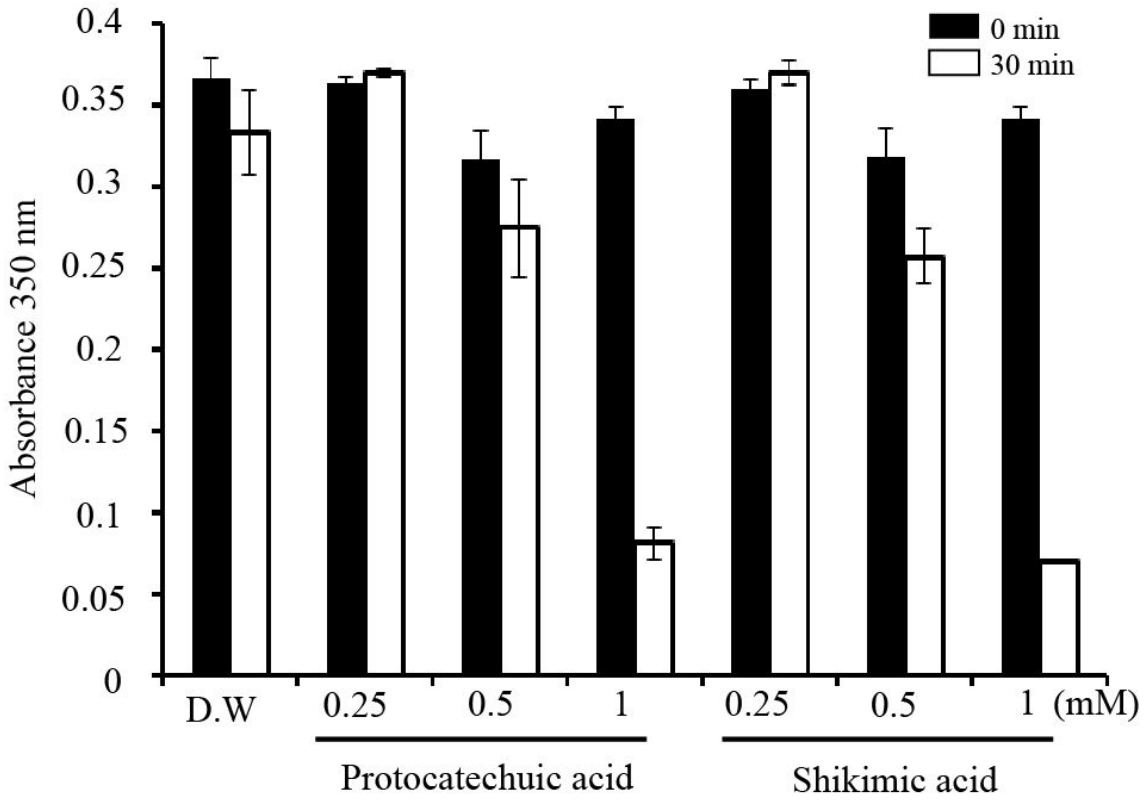

Figure 6. Fibinolysis effects of protocatechuic and shikimic acids in finbrin solution. Fibrin clots were made by adding thrombin to fibrinogen and inubating for 12 h. After fibrin clot formation, their solutions were incubated with protocatechuic and shikimic acids (0.25 - 1 mM) for 30 min. The fibrinolysis activity was determined by measuring turbidity with a UV spectrophotometer. Distilled water was used as a negative control.

Because PCA and SA strongly decreased the turbidity of fibrin clot solution, we further evaluated their fibrinolysis activity with varying pH. To determine the optimal pH for dissolving fibrin clots, the pH of solution of PCA and SA was varied from 2 to 7 before the assessment. The fibrin clot solution turbidities were measured from pH 2 to 7 using a UV spectrophotometer, and the concentrations of PCA and SA were fixed to 1 mM. Upon adding PCA and SA to the fibrin clot solution, the turbidity decreased in solutions of pH 2, 3 and 4, while the turbidity did not change in solutions at pH 5, 6 and 7 (Fig. 5A). The fibrinolysis effects of PCA and SA in test tubes after 30 min of incubation in fibrin solution were demonstrated visually. It could be seen that the fibrinolysis by the two chemicals occurred completely in the pH range of 2-4, while turbid solutions were clearly observed in the pH range of 5-7 (Fig. 5B). These results suggest that the activity of PCA and SA is influenced by pH, and the optimal fibrinolysis activity pH was determined to be in the acidic range 2-4 pH range.[42,43] The rate of fibrinolysis was also measured using a spectrophotometer at pH 4 with 1 mM of PCA and SA, respectively. PCA showed the higher rate than SA, which indicates PCA has a higher activity than SA (Fig. 6). The clear pH dependence of the activities of both compounds suggested the clue to the active forms of PCA and SA that display the fibrinolytic activity. The pKa values of PCA and SA are 4.48 and [5.19], respectively. Thus, both compounds will exist predominantly as a non-ionized form at pH 4 or lower. It is highly suggestive that it is the conjugate acid form of PCA and SA that displayed such fibrinolytic activities.

A

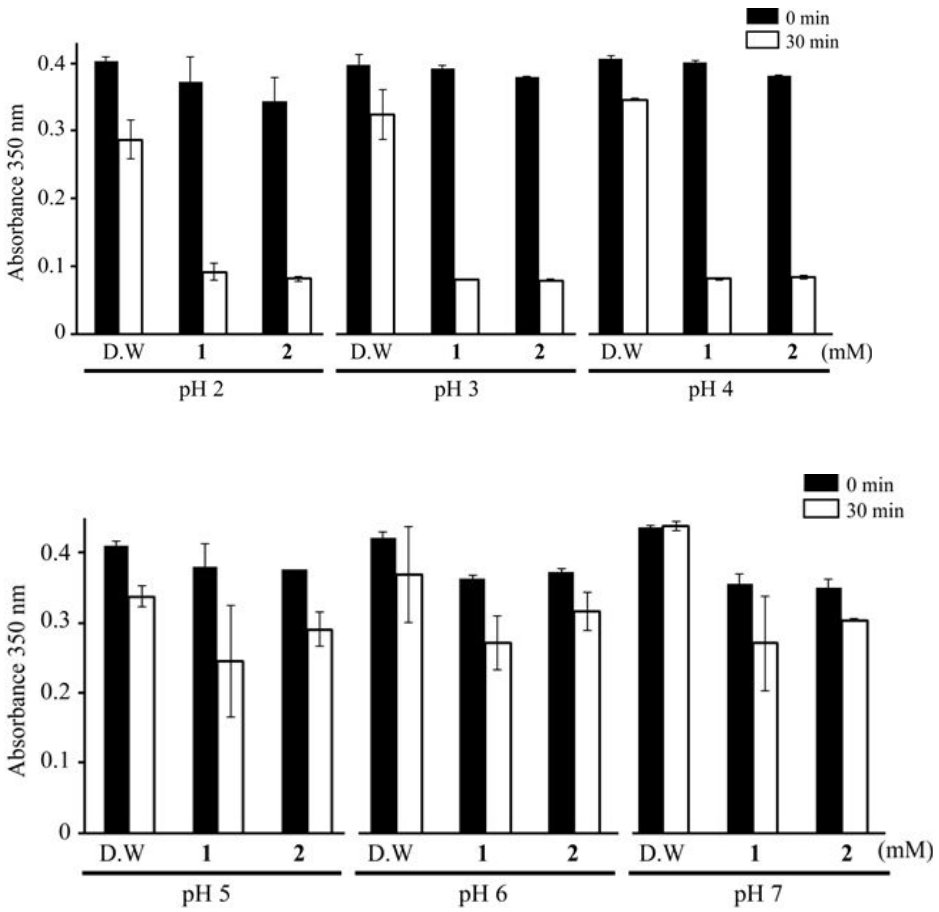

Figure 7. Changes of fibrinolysis activity by varying the pH of protocatechuic and shikimic acids in fibrin solution. A. The fibrinolysis activity of chemicals 1 and 2 (1 mM) was measured with UV (at 350 nm) after 30 min incubation. In the pH 2 ~ 4 range, the fibrinolysis mediated by the two chemicals occurred completely in the fibrin solution. In the pH 5 ~ 7 range, the fibrinolysis activity of the two chemicals decreased in the fibrin solution. B. The fibrinolytic effects of protocatechuic and shikimic acids in test tubes after 30 min incubation in fibrin solution were demonstrated visually.

B

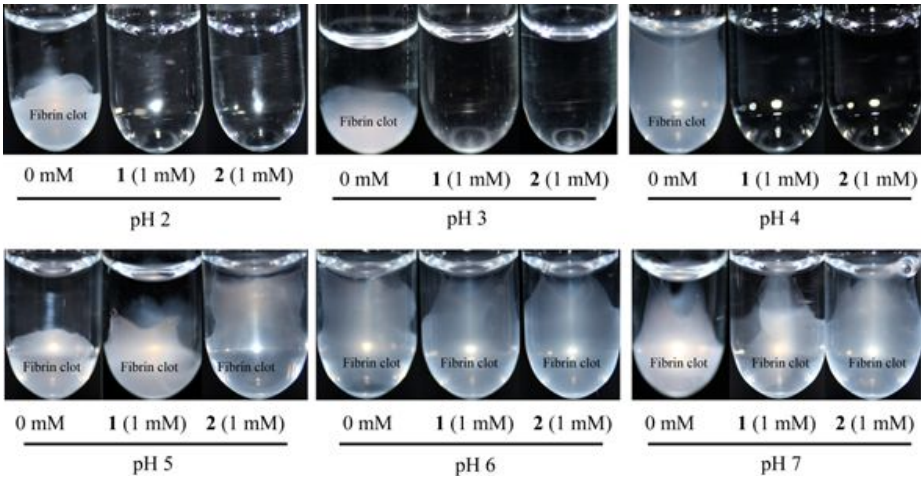

Figure 7. Changes of fibrinolysis activity by varying the pH of protocatechuic and shikimic acids in fibrin solution. (Continue)

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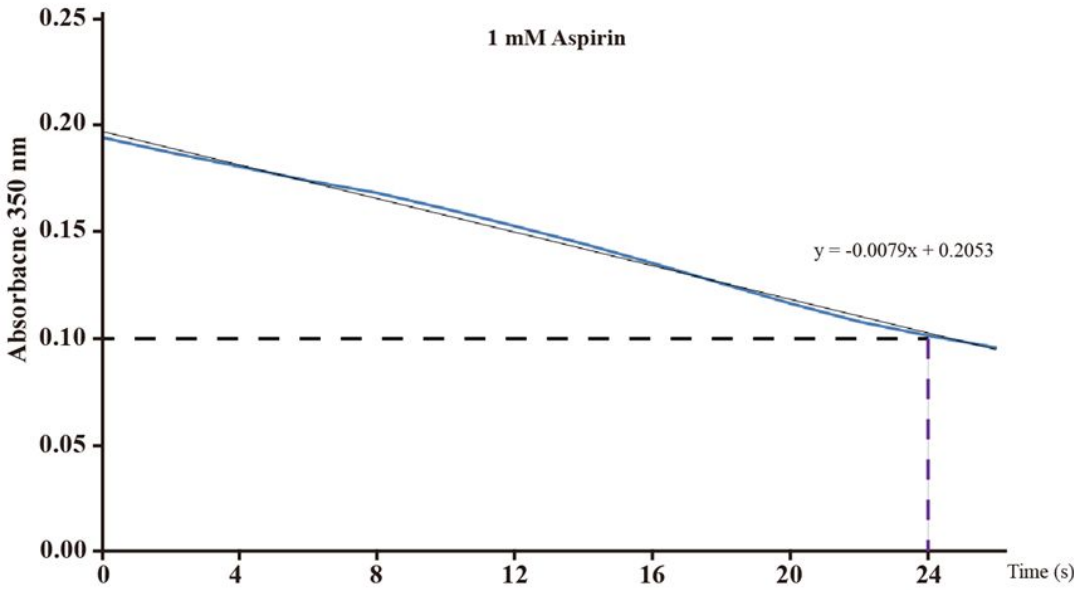

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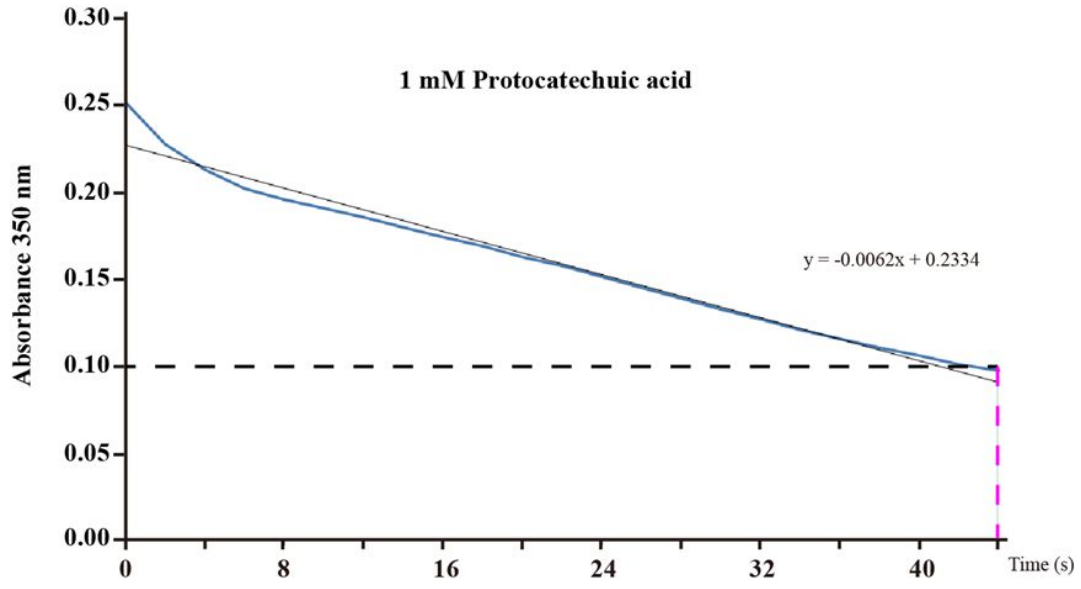

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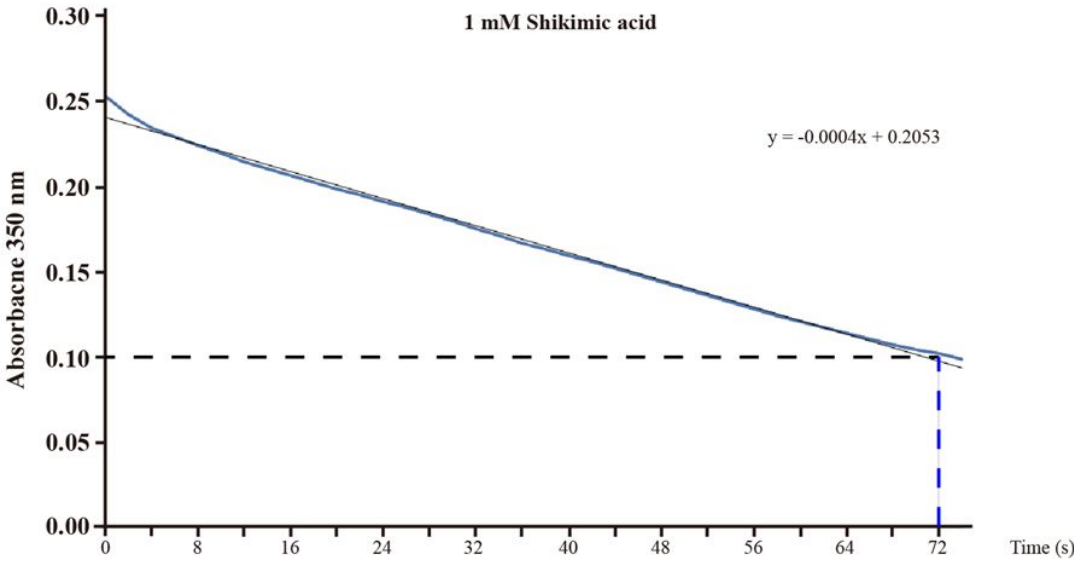


Figure 8. Time dependent change of turbidity of fibrin clots upon addition of protocatechuic acid and shikimic acid. A. The turbidity of fibrin solution was monitored in the presence of 1 mM protocatechuic acid for 44 seconds at 2 second intervals. R^2= 0.9772. B. Time dependent turbidity change of fibrin solution in the presence of 1 mM shikimic acid was monitored for 74 seconds at 2 second intervals. R^2= 0.9954

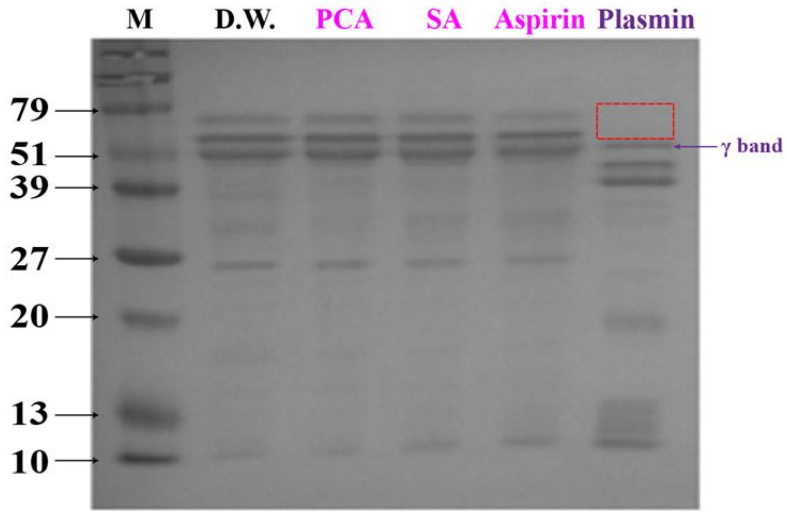
1.3.3 Fibrin and fibrinogen lysis pattern of protocatechuic acid and shikimic acid

It is well known that fibrin and fibrinogen are hydrolyzed by plasmin (Pizzo et al., 1973; Izzo et al., 1972). To compare the mode of fibrinolysis of PCA, SA, aspirin and plasmin, the changes in fibrin and fibrinogen subunit structure have been analyzed by electrophoretically examining the degraded products (in a solution that was treated with PCA, SA, and aspirin compared with plasmin). Under reducing conditions, SDS-PAGE analysis demonstrated Aα, Bβ, and γ fibrinogen chains. In the case of PCA, SA, and aspirin, the Aα, Bβ, and γ fibrin and fibrinogen chains were intact while plasmin digestion demonstrated that the Aα and Bβ fibrin and fibrinogen chains disappeared while the γ chain persisted (Fig. 7). The results demonstrated in Figure 5 suggest that similar to aspirin, PCA, SA, and aspirin do not affect polypeptide chains of fibrin and fibrinogen while plasmin directly digests the Aα and Bβ fibrin and fibrinogen chains (Francis et al., 1980; Pizzo et al., 1973; Izzo et al., 1972). Therefore, it is more likely that the fibrinolysis by PCA and SA arises from dissolving effect rather than degradation.

It was suggested that aspirin enhances fibrinolysis by acetylating fibrinogen. Acetylation of fibrinogen acetylation makes the peptide chain network more porous, which is more susceptible to fibrinolysis (Antovic et al., 2005; Fatah et al., 1996; Williams et al., 1998). However, both PCA and SA do not have any functional group that can modify a polypeptide chain. Subsequently, unlike aspirin, PCA and SA cannot exert their fibrinolytic activity via the acetylation

mechanism. The pH dependent activity suggests that the non-ionized forms in both cases are active agents. Non-ionized forms can afford more hydrophobic interactions such as a π-π stacking interaction. These binding events are expected to disrupt stabilizing interactions between polypeptide chains leading to loosening the fibrin network, and thereby induce a dissolving effect. The presence of negative charges may hinder the formation of such hydrophobic interactions. Non-ionized forms of PCA and SA still have polar functional groups such as a carboxyl group and multiple hydroxyl groups. These polar groups also may contribute additionally to the binding of these compounds to the fibrin peptide chain via hydrogen bonding. The contributions from the combination of hydrophobic interactions and hydrogen bonding to the binding of small molecules to proteins are well documented in enzyme-substrate binding.[45] Therefore, both hydrophobic interaction and hydrogen bonding from the PCA and SA are likely to disrupt ure of the peptide chain, leading to fibrinolysis.

A



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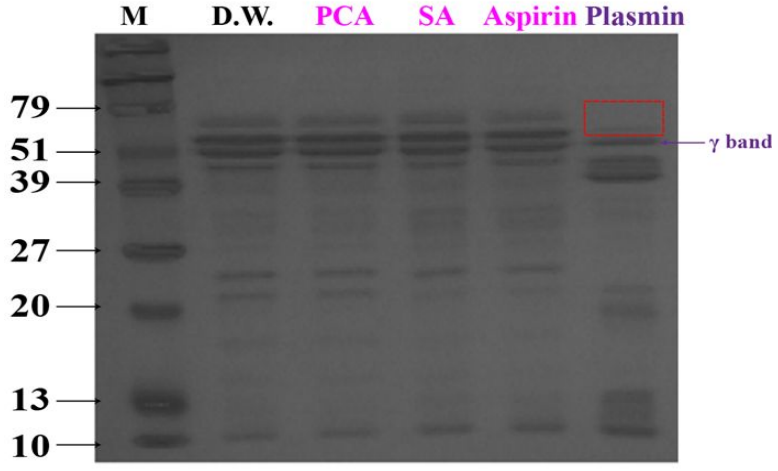


Fig. 9. SDS-PAGE fibrinolysis patterns in fibrin and fibrinogen solutions that were treated with protocatechuic acid, shikimic acid, and aspirin compared with plasmin. A. Electrophoretic patterns of fibrin on a 12% gel with protocatechuic

acid, shikimic acid, aspirin, and plasmin (M; marker, lane 1; water, lane 2; PCA, lane 3; SA, lane 4; aspirin, lane 5; plasmin). B. The electrophoretic patterns of fibrinogen on a 12% gel with protocatechuic acid, shikimic acid, aspirin, and plasmin (M; marker, lane 1; water, lane 2; PCA, lane 3; SA, lane 4; aspirin, lane 5; plasmin).

1.3.4 Antithrombotic and bleeding time effects of protocatechuic and shikimic acids in mouse

To test the antithrombolytic activity of protocatechuic and shikimic acids in vivo, carrageenan-induced mouse tail thrombosis model and collagen plus epinephrine induced pulmonary thromboembolism model of mice were used. Carrageenan (1 mg/kg) was dissolved in saline and was an intravenous injection. The lengths of the infarct region of the mouse tails were measured after 1, 3, 6, 12, 24, and 48 h treatment. Only when injected saline, swelling and reddening or bluing of tail was seen first and then became auburn. After 48 h, the infarct regions of the tail were found to significantly increase with time. However, Aspirin, PCA, and SA were showed that the infarct regions of the tail were decrease or nearly disappeared after 48 h treatment with 100 mM (Fig. 8). It could be conclude in Figure 8 that PCA and SA significantly inhibited carrageenan-induced mouse tail thrombus formation in vivo. It was also concluded that the remaining time of three chemical in the mouse tail was increased antithrombosis activity. The representative actual results of each group at 6 h were showed in Figure 9. The average thrombus length of aspirin, PCA, and SA was 3.85, 3.03, and 3.60 cm, respectively. The thrombus nearly disappeared in the tip of mouse tail in aspirin, PCA, and SA. From these results, it was concluded that three chemicals could significantly inhibit thrombus formation in carrageenan induced mouse tail thrombus model. To antithrombotic effect of PCA ans SA in collagen and epinephrine induced acute pulmonary thromboembolism model, a mixture of collagen

and epinephrine into the tail vein of mice was intravenous administration. Vehicle caused death or paralysis in 100% mice within 15 min. Intravenous adminstration of 100 mM PCA and SA survived 80% mice, but survival rate of 100 mM aspirin was 50% (Table 1).

In all mice, although small thrombi was seen partly the vicinity of the intracuff placement, no thrombus was present in the contralateral carotid artery as a control (Fig. 10A). The occlusive thrombus was observed at the center of the intracuff region in $FeCl_3$ treated groups at 30 min after the combined applicatin of ligation and cuff placement (Fig. 10B). FeCl3 and aspirin groups were shown to half region (Fig. 10C). In contrast, no thrombus was seen at PCA and SA groups (Fig. 10D and E). To study the effect on hemostasis, the tail bleeding assay was performed. Tail bleeding times of control mice were 515 sec. However, 100 mM PCA and SA with strong thrombolytic effect produced a mild nearly disappeared or little increase (1.0 and 1.1 fold, respectively) in bleeding time than control mice. On the other hand, 100 mM aspirin increased the bleeding times 3.6 fold over control mice (Fig. 11). In most mouse models of thrombus formation, thrombus was rapidly generated from within a few seconds. (Carmeliet 1997, Farrehi 1998, Roesken 1997, Hara 1991, Roque 2000) These models by means of acute and dramatic injury of endothelium appear not to be suitable for investigating the course and mechanism of endothelium damage or injury, since it is too acute to detect the pathophysiological changes of endothelium after stimuli. This model is likely to be of particular value in studying the relationship between thrombosis formation and the alteration of

endothelial cell morphology and function in detail. An endothelial site-specific disruption induced by the combination of ligation and cuff placement provides a unique model for spontaneous thrombosis secondary to endothelial cell damages. Therefore, this model provides insight into the physiological role and importance of endothelial function in vivo and constitutes a valuable animal model for the evaluation of new therapeutic strategies for the treatment of thrombogenesis (Sasaki 2004). Sasaki et al. had confirmed that aspirin inhibited of the thrombus formation. In this study, new function of PCA and SA was found that could inhibit carrageenan-induced tail thrombosis. Also, the protective effect of PCA and SA was observed in the collagen and epinephirine-induced pulmonary thromboembolism mice model. The antithrombotic effects in FeCl3 induced carotid arterial thrombus model showed that PCA and SA attenuate thrombosis. Although the mechanism of antithrombus by PCA and SA is not study in detail, the data of in vivo thrombosis models might provide significant information for research.

In conclusion, a strong fibirnolysis chemical, PCA and SA were purified from fermented pine needle extract and biochemical activity of PCA and SA was investigated from various view. PCA and SA had ability to dissolve fibrin and fibrinogen. Unlike fibrinolytic protease, PCA and SA displayed uncommon cleavage pattern of human fibrin and fibrinogen. The thrombolytic abilities of PCA and SA in vitro and in vivo with lower hemorrhagic risk make it a promising candidate in thrombolytic theraphy. Further studies are needed to understand toxic effects of PCA and SA, mechanism of action at molecular level and to

optimize its dose, and duration to improve the outcomes in various in vitro and in vivo models.

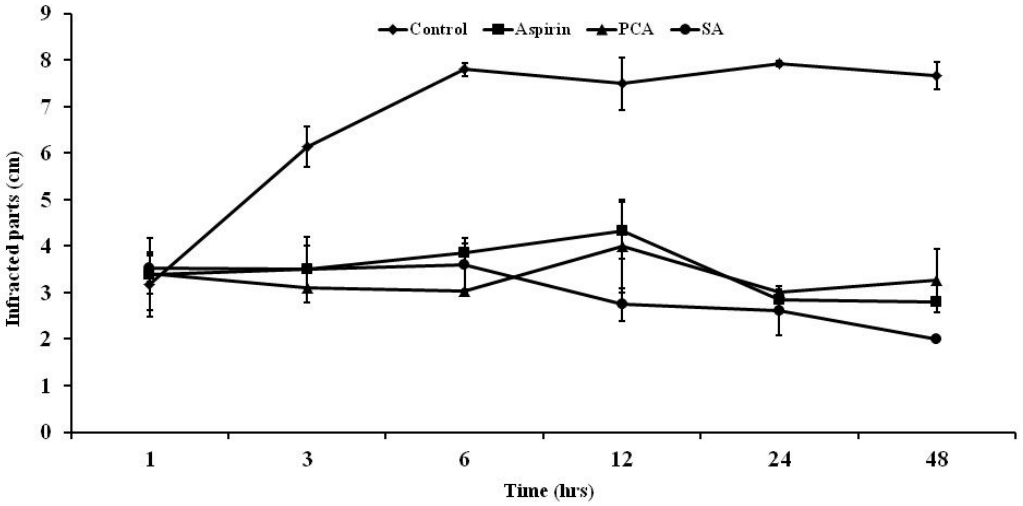
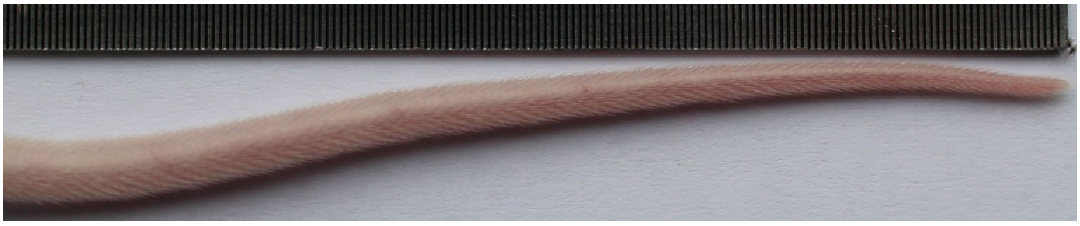


Fig. 10. The effects of protocatechuic and shikimic acids on carrageenan induced mouse tail thrombus length. The analysis is based on three indepentent experiments. All mouse were induced thrombosis by carrageenan. Control is saline treatment. Concentration of aspirin, protocatechuic and shikimic acids is 100 mM.

A



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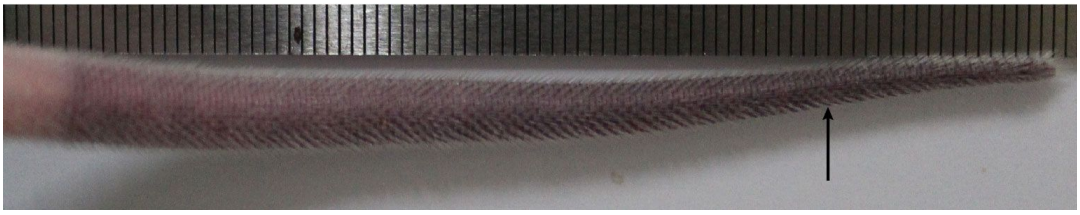

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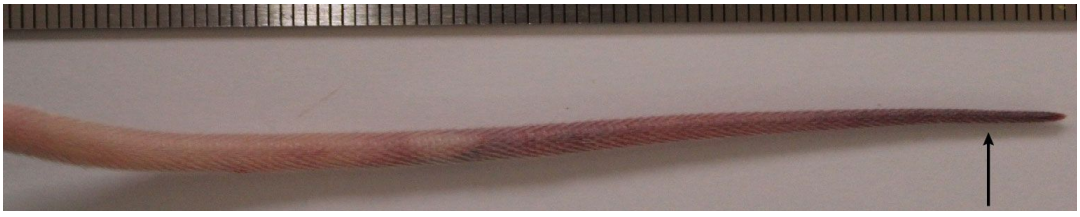

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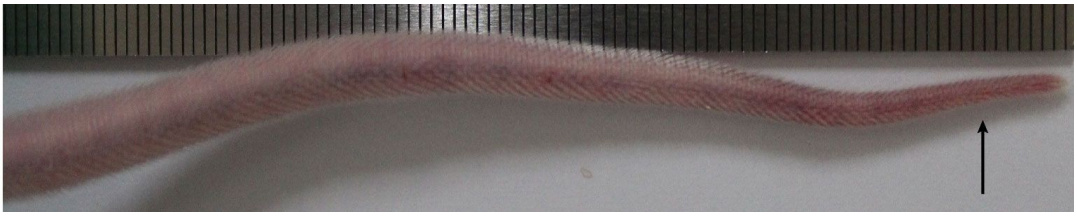

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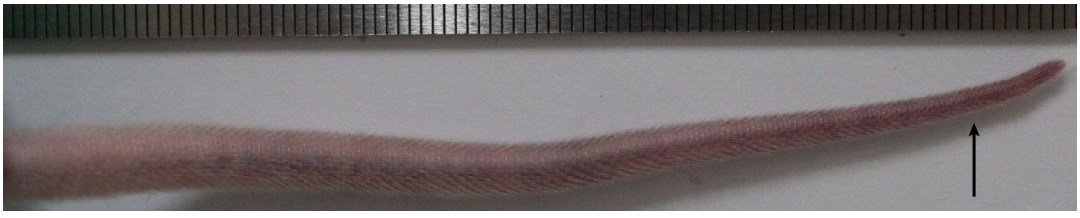


Fig. 11. The effect of protocatechuic and shikimic acids on carrageena induced mouse tail thrombus length (6h after carrageenan injection). Effects of Asprin, protocatechuic, and shikimic acids on carrageenan-induced thrombus formation in mice after 6 h. Length of mice tail thrombosis with saline (A), carrageenan (B), carrageenan and 100 mM Aspirin (100 uL) (C), carrageenan and 100 mM protocatechuic acid (100 uL) (D), carrageenan and 100 mM shikimic acid (100uL) (E). Arrows indicate thrombus formation region (wine-colored). Data represents one of three experiments.

Table 1. Effect of protocatechuic and shikimic acids on pulmonary thromboembolism induced by collagen and epinephrine in mice.

Experimental group	Protection (%)	No. paralyzed or dead / No. tested
Vehicle	0	10/10
PCA (100 mM, 2ml treat)	80	2/10
SA (100 mM, 2ml treat)	80	2/10
Aspirin (100 mM, 2ml treat)	50	5/10

Mice were injected with indicated concentrations of aspirin, protocatechuic and shikimic acids. After 15 minutes. the mixture of collagen and epinephrine was injected and the number of killed or paralyzed mice was recorded.

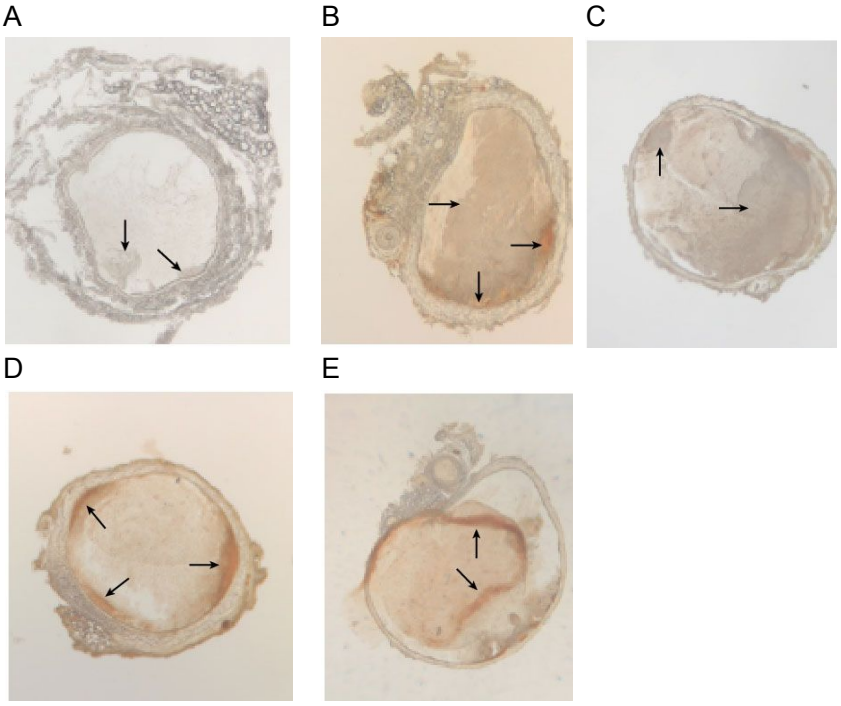


Fig. 12. Antithrombotic effect of aspirin, protocatechuic and shikimic acids in $FeCl_2$-induced carotid arterial thrombus model. Effects of aspirin, protocatechuic and shikimic acids on $FeCl_3$-induced carotid arterial thrombus formation in rat. The occlusion of the carotid artery with ferric chloride (B), ferric chloride and aspirin (C), ferric chloride and protocatechuic acid (D), ferric chloride and shikimic acid (E), compared with untreated control (Vehicle) (A). Arrows indicate thrombus formation region. Data represents one of three experiments.

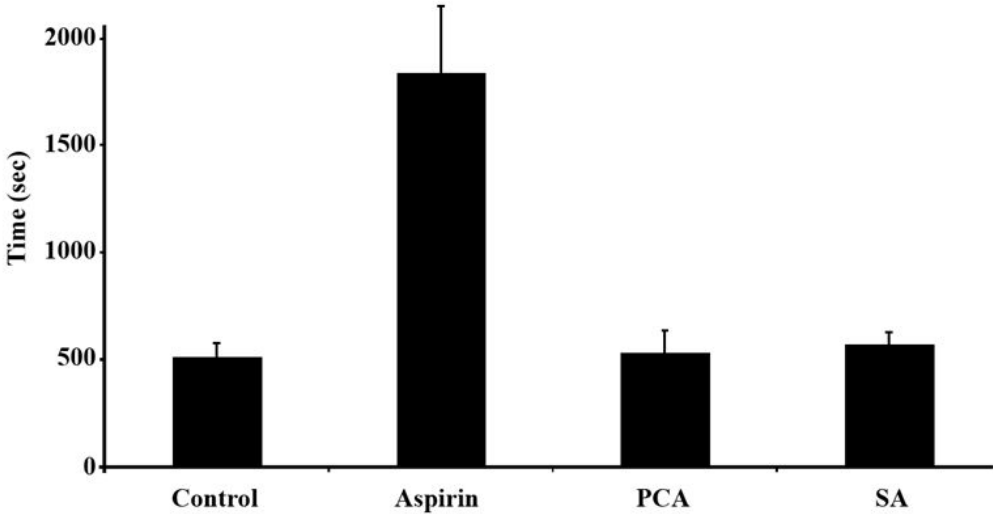


Fig. 13. Effect of protocatechuic acid and shikimic acid in bleeding time. Control (saline), aspirin, protocatechuic and shikimic acids were inhedted in the tail veins of mice 30 min before the bleeding time and blood volume were recorded. Each value is expressed as mean ± SD of at least three independent experiments.

With the above results in hand, we performed HPLC analysis to determine the yield of PCA and SA in the FPEs. The amounts of chemical PCA in the EtOAc- and SA in the *n*-BuOH-soluble fraction of the FPEs were calculated from the HPLC peak area. The amounts of chemicals PCA and SA were 19.1 (± 2.80) μg/mL and 300.07 μg/mL, respectively, in FPE 0, which is the starting pine needle extract fermentation. The amount of chemical PCA gradually increased to 27.8 (± 0.16), 41.6 (± 0.40) and 79.1 (± 9.91) μg/mL during 3 years of fermentation, while the amount of chemical SA did not demonstrate large changes after PCA year of fermentation (FPE1 418.60 ± 1.12; FPE2 371.79 ± 2.7; FPE3 372.93 ± 6.90 μg/mL) during the same fermentation period (Fig. 8). The level of acidic metabolites from several microorganisms and algae were studied under a variety of incubation conditions (Barrero, 1996). In phycomyces, a noticeable increase in chemical PCA content was observed together with a decrease in gallic acid.[46] All of the phenolic acid levels decreased during the winemaking process, whereas only gallic acid increased (Borazan et al., 2013; Ginjom et al., 2011; Ivanova et al., 2011). Although a considerable number of studies on phenolic acids have been performed, there has always been a challenge due to the complexity and diversity of these compounds.

SA is a precursor for the synthesis of oseltamivir phosphate (Tamiflu), which is used as an anti-viral for the H5N1 (bird flu) virus strain (Sui, 2008). However, the availability of SA is limited compared with its tremendous demand (Sui 2008; Johansson et al., 2005). Therefore, pine needles are an excellent resource to provide inexpensive and sufficient quantities of compound SA for industrial-scale

extraction (Sui 2008). While the quantity of SA remained unchanged during the fermentation processes, the research is underway, and the explanation for the lack of a change in assay quantity will be published later in a separate publication.

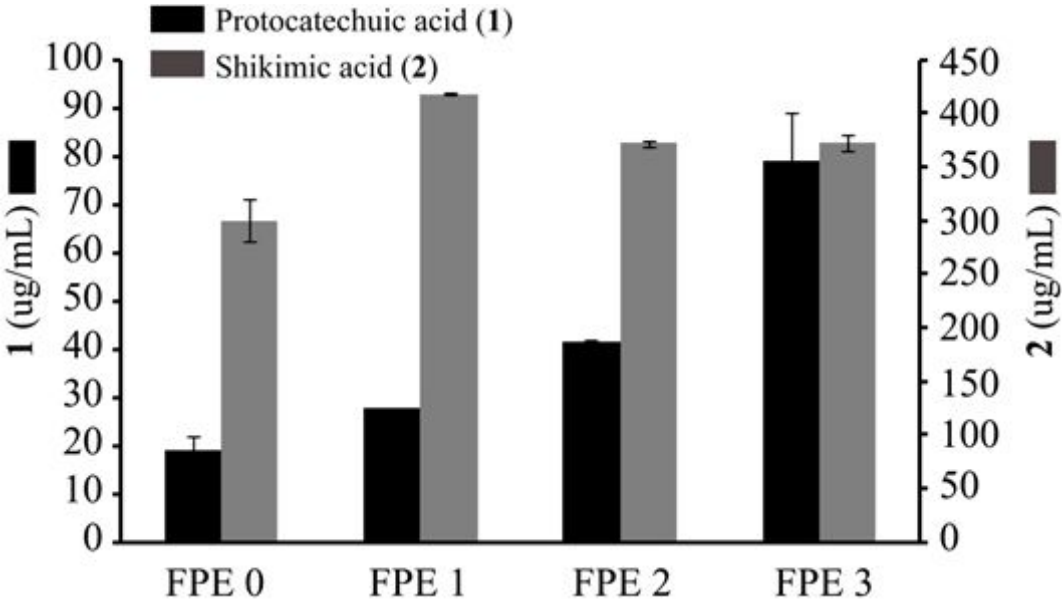


Fig. 14. Quantitative analysis of protocatechuic and shikimic acids obtained from fermented pine needle extracts (FPE). A. Protocatechuic acid quantity changed from 19.13 (FPE 0) to 27.08 (FPE 1), 41.6 (FPE 2), and 79.07 (FPE 3) μg/mL as fermentation proceeded. B. Shikimic acid quantity changed from 300.07 (FPE 0) to 418.6 (FPE 1), 371.79 (FPE 2), and 372.93 (FPE 3) μg/mL as fermentation proceeded. The quantity of protocatechuic acid increased steadily, while the quantity of shikimic acid did not change.

Chater 2.

Screening of compounds with biological activity on angiotensin I converitng enzyme inhibition from *Pinus densiflora*

2.1 Introduction

Cardiovascular disease (CVD) is reponsible for one-third of global deaths and is a leading and increasing contributor to the global disease burden (Diane et al., 2002). Importantly, CVD is eminently preventable. In order to achieve significant reductions in the avoidable CVD burden, a combination of population-based and high-risk strategies necessary. These strategies should target lifestyle-related risk factors such as unhelathy diet, physical inactivity and tobacco use, as well as the intermdiate mainfestations of these lifestyles; hypertension, glucose intolerance, and hyperlipidemia. In addition, strategies aimed at improving management of those already affected by CVD should be an integral component of a comprehensive approach for the prevention and control of CVD.

Hypertension is already a highly prevalent risk factor for CVD throughout the industrialized world. It is becoming an increasingly common helath problem worldwide because of increasing longevity and prevalence of contributing factors such as obesity, physical inactivity and an unhealthy diet (Wada et al., 1985; Savluchinsake et al., 1997)). The current prevalence in many developing countries, particularly in urban societies, is already as high as those seen in

developed countries (Tolmacheva et al., 2006; Tagat et al., 1994). Hypertension plays a major etiologic role in the development of cerebrovascular disease, ischemic heart disease, cardiac and renal failure. Treating hypertension has been associated with about a 40% reduction in the risk of stroke and about a 15% reduction in the risk of myocardial infarction (Kinouchi et al., 2000). Although the treatment of hypertension has been shown to prevent CVD and to extend and enhance life, hypertension remains inadequately managed everywhere (Prinz et al., 2002; Vidotti et al., 2004; Yoon et al., 2000; Bernsterin et al., 1989; Acharya et al., 2003; Ehlers et al., 1989; Unger 2002). In addition, hypertension often coexists with other cardiovascular risk factors, such as tobacco use, diabetes, hyperlipidemia and obesity, which compound the cardovascular risk attributable to hypertension (Prinz et al., 2002; Vidotti et al., 2004; Yoon et al., 2000).

Diterpene resin acids are an important compound to prevent herbivores and pathogens (Diane et al., 2002). The biological activity of diterpene resin acids, such as antimicrobial, antiulcer, and cardiovascular activityes is the most represented for this class of diterpenoids. Dehydroabietic acid and its derivatives exhibit antiulcer antiulcer (Wada et al., 1985), antimicrobial (Savluchinske et al., 1997), anxiolytic (Tolmacheva et al., 2006) antiviral (Tagat et al., 1994), antitumor (Kinouchi et al., 2000), and cytotoxic activities (Prinz et al., 2002).

2.2 Material and Method

2.2.1 General experimental procedures

UV spectra were recorded in MeOH on a UV-vis spectrophotometer (Optizen, 3220UV). NMR spectra were attained using a Varian Unity-INOVA spectrometer that was operated at 500 MHz for 1H NMR and at 125 MHz for 13C NMR with complete proton decoupling at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). The deuterated solvent for NMR analysis (CDCl3) was purchased from CIL (Cambridge Isotope Laboratory, USA). The MS data were obtained using a Micro mass QTOF2 (Micro mass, Wythenshawe, UK) mass spectrometer. Silica gel (Merck, 63 - 200 um particle size) and C-18 silica gel (Merck, 75 um particle size) were used for column chromatography. TLC was obtained using silica gel 60 F254 and RP-18 F254 plates that were purchased from Merck. To isolate compounds 1 and 2, HPLC was performed using a Gilson (Middleton, USA) system with a UV detector and an ODS-H80 column (20 × 150 mm, 4 μm particle size). To quantify the two compounds, a reversed-phase HPLC with a pack polymer C18 column (6 μm particle size, 4.6 mm, 250 mm) from the YMC Corporation, and a Shimadzu HPLC system (Tokyo, Japan) consisting of a LC-20AT pump, CTO-10AS column oven, and an SPD-20A UV detector was employed. To measure angiotensin I converting enzyme assay, an Eon UV spectrophotometer from BioTek Instruments, Inc. (Winooski, USA) was used.

Software was using Gen 5.2. Hippuric aicd, angiotensin converting enzyme from rabbit lung, captopril, and N-Hippuryl-His-Leu were purchased from the Sigma Aldrich Co. (St. Louis, USA). Compounds for the assay were dissolved at a concentration of 50 mM in borate buffer (pH 8.3) containing 300 mM NaCl. All of the solvents used for extraction and isolation were of analytical grade.

2.2.2 Plant material

Fresh red pine needles (Pinus densiflora Sieb. et Zucc.) were picked up from red pine in the Gokseong province, Jeollanam-Do, Southern Korea. Harvested red pine needles were cleaned with tap water (cleaned with 5% charcoal) and dehydrated with the spin-drier.

2.2.3 Extraction and fractionation

The dried red pine needles (Pinus densiflora Sieb. et Zucc.) (49 kg) was exhaustively extracted with 70% metanol. The dried and powdered pine needles extractwas dissolved in H_2O (1.0 L) and then partitioned (1:1) with *n*-hexane to give an n-hexane-soluble fraction. The H_2O layer was dried and H_2O (500 mL) was then added. The aqueous solution was partitioned with ethyl acetate (EtOAc) to separate the EtOAc-soluble fraction (500 g) from the H_2O-soluble fractions. The EtOAc-soluble fraction (500 g) was passed over a silica gel column and eluted with CH_2Cl_2:MeOH (30:1 to 0:1) to give ten fractions (F1-F10).

The F2.7.1 fraction was loaded onto an HPLC Optimapak C18 column to yield dehydroabietic and commnuic acids using a mixed solvent of MeOH-H_2O (80:20) as an eluent.

2.2.4 Measurement of antihypertensive activity using an Angiotensin converting enzyme spectrophotometer assay

The spectrophotometric assay for angiotensin converting enzyme (ACE) was performed with a modified method of Cushman and Cheung (1970). Incubations for hippuryl-his-leu (HHL) hydrolysis by ACE were carried out for 30 min at 37℃. Each 150 uL assay mixture contained the following components at the indicated final concentration: 100 uL of barate buffer (100 mM pH 8.3 containing 300 mM NaCl and 5 mM HHL) and 150 uL of the enzyme. The enzyme was added last to initiate the reaction. An aliquot of 60 uL of diluted sample or distiled water was added to the sample mixture or control mixture, respectively. The enzymatic reactions were terminated by addition 250 uL of 1 N HCl; the HCl was added before the enzyme in zero-time all blank assays. The hippuric acid formed by action of the ACE on HHL was extracted from the acidified solution into 1.5 mL of ethyl acetate by vortex mixing for 15 sec. After a brief centrifugation, a 1.0 mL aliquot of each ethyl acetate layer was transferred to a clean tube. The ethyl acetate aliquots were evaporated by heating at 60℃ for 1 h in a baccu pump.

The hippuric acid was redissolved in 1.0 mL of distilled water and the amount of hippuric acid was determined from its abosrbance at 228 nm. The ACE inhibition rate was calculated as follow:

$$\text{ACE inhibition rate (\%)} = \frac{(B-A)}{(B)} \times 100$$

where A and B are the test samples without and with compound or fraction.

2.2.5 Cell culture

The simian virus 40 transfected mouse lymph node endothelial cell line SVEC4-10, was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured following ATCC guidelines. Cells were maintained in DMEM with 4.5 g/L glucose and 10% FCS and 1% antibiotic and culture medium renewed every 48 hours.

2.2.6 Cell treatments and cytotoxicity

SVEC 4-10 cells were clutured in DMEM, containing 100 ug/mL penicillin-streptomycin, 10% foetal bovine serum (FBS) and maintained at 37℃ under a humidified atmosphere with 5% CO_2. Cytotoxicity levels of the test samples on SVEC 4-10 cells were measured using MTT (3-(4,5-dimethyl-2yl)-2,5-dipheyltetrazolum bromide) as described by Hanse, Nielson, and Berg (1989), with slight modifications.

Cells were cultured in 96-well plates at a density of 1 × 10^6 cells/well. Cells were washed with fresh medium and were treated with different concentrations of test samples following 48 h of incubation. Then cells were washed with phosphate buffered saline (PBS) and 100 mL of MTT solution (1 mg/mL) were added to each well and kept for 4 h. MTT solution in each well was removed and 100 mL of dimethyl sulfoxide (DMSO) were added to solubilise the formed formazan crystals. The optical density was measured at 540 nm using a UV microplate reader (Tecan Austria GmbH, Groedig, Austria). Relative cell viability was calculated compared to the non-treated blank group. The data were expressed as means of at least three independent experiments. Furthermore, the effect of LPS treatment on the viability of SVEC cells were also analysed by MTT assay.

2.2.7 Total RNA Isolation and cDNA synthesis

After incubation, the cells were washed twice with phosphate buffered saline (PBS) and the total mRNA was isolated using GeneAll kit according to the manufacturer's protocol. cDNA synthesis was performed using total RNA and the Superscrit First-strand synthesis syste, also according to the manufacturer's established protocaol (Invitrogen, Carlsbad, CA). Thereafter, it was reverse-transcripted as following program: 25℃ 10 min, 42℃ 1 h, 72℃ 15 min, and the cDNA product was stored at - 80℃.

2.2.8 Reverse transciption polymerase chain reaction

Total RNA was isolated with Hybrid-R kit (GeneAll). 1 ug of total RNA was used to synthesis cDNA using oligo dT 18 mer primers with the Hyperscript Reverse transcriptase kit (GeneAll). RT-PCR amplification was performed with standard method and products were examined by gel electrophoresis on 1% agarose gels. The used primers was as fllows; Angiotensin II receptor F and R, eNOS F and R, beta-actin F and R.

2.3 Results and discussion

2.3.1 Isolation and purificaiton of antihypertension compounds

The pine needle extract (FPE) demonstrated strong fibrinolysis activity.27,28 The FPE with fibrinolysis activity was fractionated with *n*-hexane, ethyl acetate-(EtOAc). Among two fractions, the EtOAc soluble fractions exhibited strong antihypertension activity. The EtOAc-soluble fraction that exhibited strong antihypertension activity was further purified by repeated silica gel, RP C 18 column/HPLC (Fig. 13). Dehydroabietic acid was obtained as a white amorphous powder. Molecular formula of dehydroabietic acid was $C_{20}H_{28}O_2$. The 1H NMR data showed four tertiary methly signals at δH 1.29, 1.22, and two 1.23, three olefinic proton at δH 7.17, 7.00, 6.89. The 13C NMR spectroscopic data displayed 20 carbon signals, two olefinic carbon at δC 134.8, 127.0, 124.3, and 1240, and a carboxylic group at δC 185.3. (Fig. 14 and Table 2). Communic acid was obtained as acrystalline form. Molecular formula of communic acid was $C_{20}H_{30}O_2$. The 1H NMR data showed two tertiary methly signals at δH 14.8 and 12.0, three olefinic proton at δH 6.33, 5.41, and 2.00. The 13C NMR spectroscopic data displayed 20 carbon signals, two olefinic carbon at δC 147.8, 141.6, 133.8, and 108.3, and a carboxylic group at δC 184.4. The chemical structures of the dehydroabietic acid and communic acid from the EtOAc-soluble fraction was identified using spectroscopic data (Fig. 15 and Table 3).

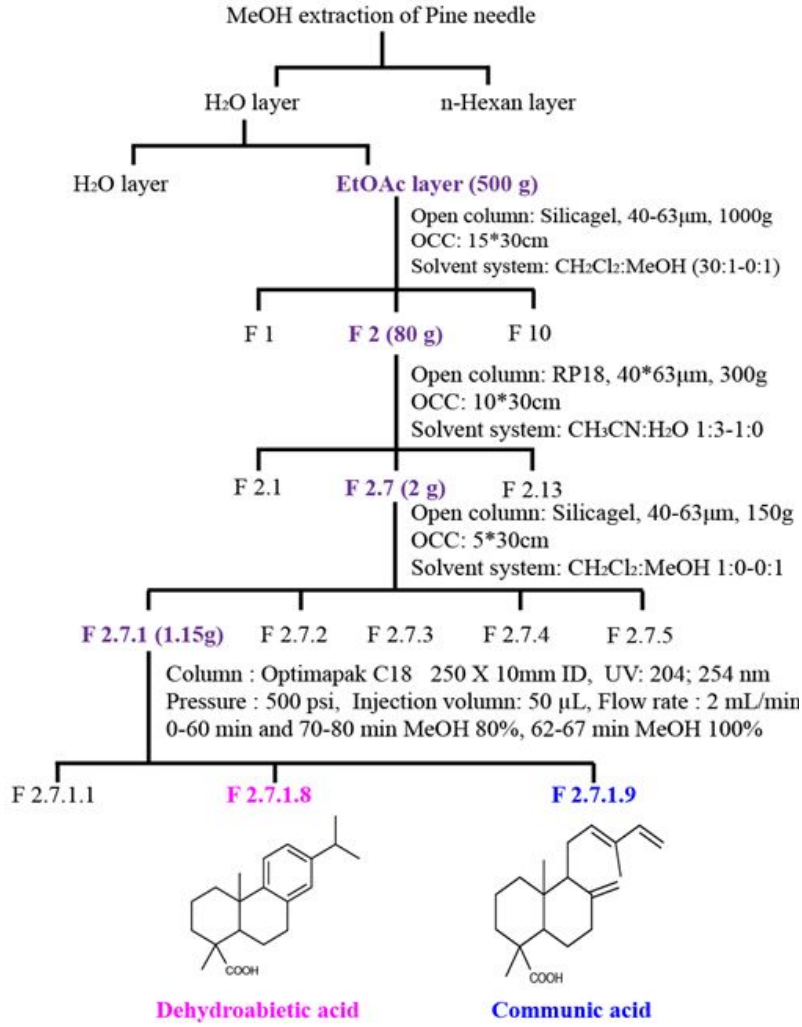
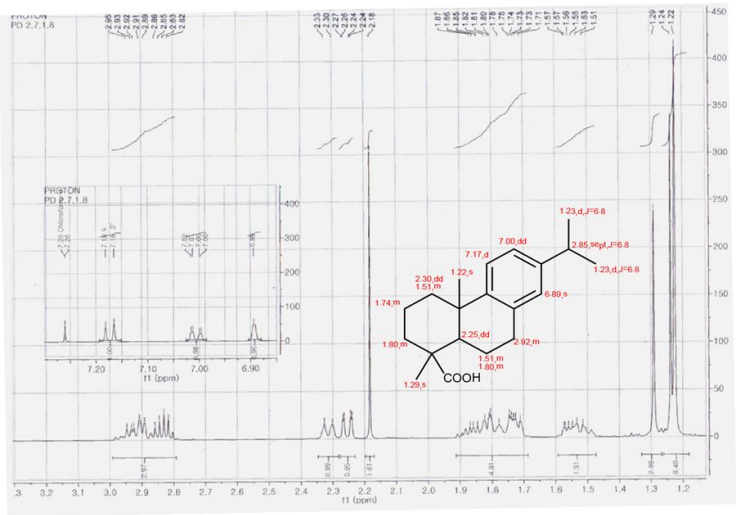


Figure 15. Procedure for islating dehydroabietic acid and communic acid from pine needle. The ethyl acetate-soluble fraction exhiting strong fibrinolysis activity was further purified by repeated silica gel column and HPLC, and the *n*-butyl alcohol-soluble fraction was purified with a Sephadex LH-20 column and HPLC. The structures of dehydroabietic acid and shikimic acid from the ethyl acetate-soluble fraction was identifed by ^{1}H NMR and ^{13}C NMR spectroscopy.

A



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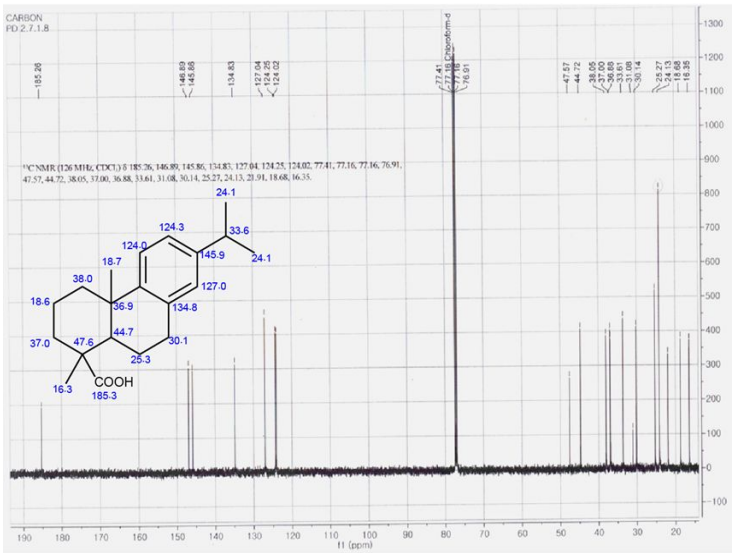
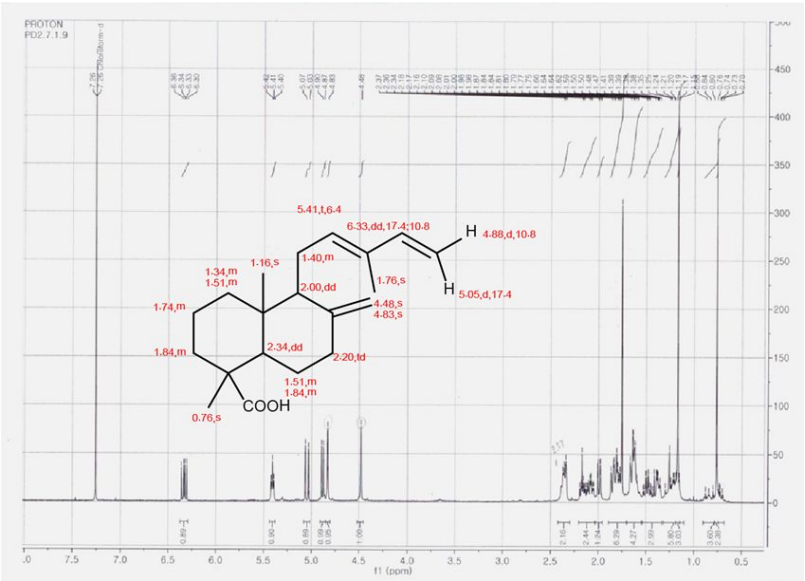


Figure 16. ^{1}H and ^{13}C NMR spectra of dehydroabietic acid isolated from pine needles. A. ^{1}H NMR spectrum. B. ^{13}C NMR spectrum.

A



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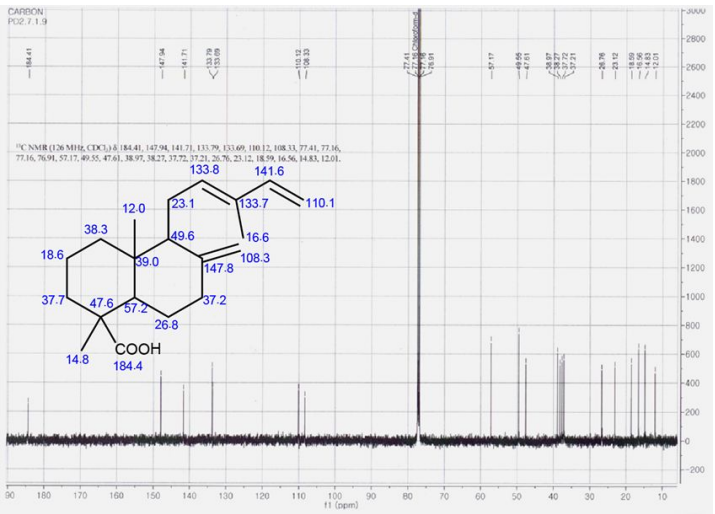

Figure 17. ^{1}H and ^{13}C NMR spectra of communic acid isolated from pine needle. A. ^{1}H NMR spectrum. B. ^{13}C NMR spectrum.

Table 2. NMR sepctroscopic data (500 MHz) for dehydroabietic acid

Postion	δH (J in Hz)	δC	
1	2.30,dd 1.51,m	38.0	CH2
2	1.74, m	18.6	CH2
3	1.80, m	37.0	CH2
4		47.6	C
5	2.25, dd	44.7	CH
6	1.51, m 1.80, m	25.3	CH2
7	2.92, m	30.1	CH2
8		134.8	C
9		127.0	C
10		36.9	C
11	7.17, d	124.0	CH
12	7.00, dd	124.3	CH
13		145.9	C
14	6.89, s	127.0	CH
15	2.85, sept,j = 6.8	33.6	CH
16	1.23, d, j = 6.8	24.1	CH3
17	1.23, d, j= 6.8	24.1	CH3
18		185.3	C
19	1.29, s	16.3	CH3
20	1.22, s	18.7	CH3

Table 3. NMR sepctroscopic data (500 MHz) for communic acid

Postion	δH (J in Hz)	δC	
1	1.34, m 1.51, m	38.3	CH2
2	1.74, m	18.6	CH2
3	1.84, m	37.7	CH2
4		47.6	C
5	2.34, dd	57.2	CH
6	1.51, m 1.84, m	26.8	CH2
7	2.20, m	37.2	CH2
8		147.8	C
9	2.00, dd	49.6	CH
10		39.0	C
11	1.40, d	23.1	CH2
12	5.41, t, 6.4	133.8	CH
13		133.7	C
14	6.33, dd, 17.4; 10.8	141.6	CH
15	4.88, d, 10.8 5.05, d, 17.4	110.1	CH2
16	1.76, s	16.6	CH3
17	4.48, s, 4.83, s	108.3	CH2
18	0.76, s	14.8	CH3
19		184.4	C
20	1.16, s	12.0	CH3

2.3.2 Angiotensin converting enzyme inhibitory activity of dehydroabietic acid and communic acid.

The ACE inhibition activity was examineed by measuring the concentration of hippuric acid liberated from hippuryl-His-Leu by the modified method of Cushman and Cheung (1970). The ACE inhibition of red pine was shown in Figure 16. The first fraction was separated using silica 63 - 200 um. The Among ten EtOAc partition, Fraction 2 was the most effective in ACE inhibition. Next, Fraction 2 was isolated using a RP C-18 (40 × 63 um). Among the second partition, Fraction 2.7 was the most effective in ACE inhibition with 63.3%. Fraction 2.7 was separated five sub-fraction using silica gel (40 - 63 um), and Fraction 2.7.1 was the most ACE inhibition activity (75.4%) and was spilted using HPLC and Opimapak C 18 column. The final partition was divided nine fraction and fraction 2.7.1.8 and 2.7.1.9 had the most inhibitory activity against ACE with 74.8% and 69.9, respectively (Fig. 16 and 17).

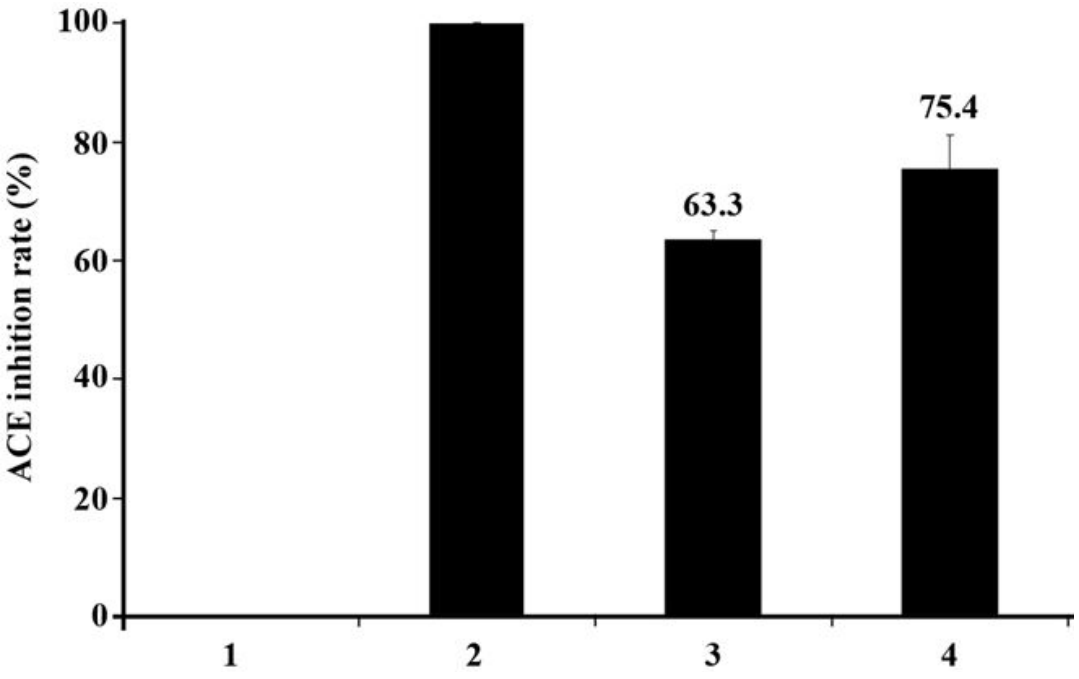

Figure 18. Angiotensin converting enzyme inhibitory effect of ethyl acetate extracts from pine needles. 1: negative control, 2: 3 mM captopril, 3: 5 mg/mL Fr.2-7, 4: 5 mg/mL Fr.2-7-1. Bars represent means ± SD (n=3).

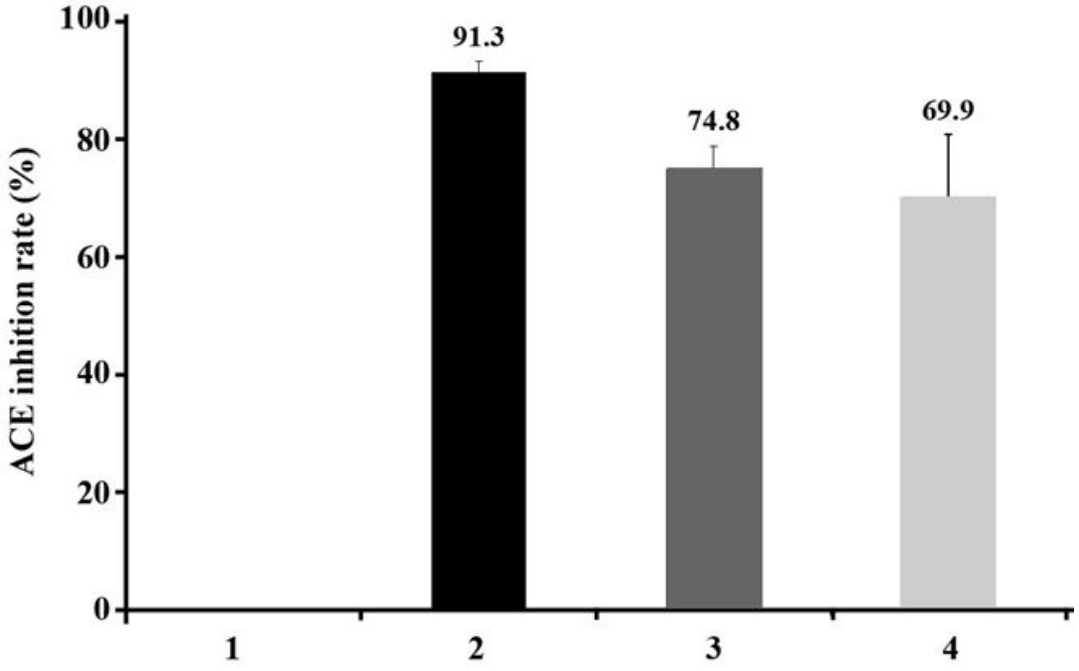

Figure 19. Angiotensin converting enzyme inhibitory effect of islated compound from ethyl acetate partions. 1: negative control, 2: 3 mM captopril, 3: 3 mM dehydroabietic acid, 4: 3 mM communic acid. Bars represent means ± SD (n=3).

2.3.3 Expression of *Angiotensin II receptor*, type 1 and *endothelial nitric oxide synthase 3* (eNOS) in mouse endothelial cells.

In order to investigate the antihypertesion activity of the purified compound , cDNA expression of *Angiotensi II receptor*, type 1 (*AT_1 receptor*) and *endothelial nitric oxide synthase 3* (*eNOS*) was identified by RT-PCR in SVEC 4-10. To identify expression of *AT_1* receptor and *eNOS* gene in SVEC 4-10, group of cells were exposed to high glucose concentration for a shorter period of 6 h. Vidotti et. al. was reported that high concentration glucose induced *AT_1 receptor* and *ACE* expression (Vidotti, 2004). As shown in Figure 18, cDNA expression of *AT_1 receptor* and *eNOS* was regulated with the treatment of communic acid. The treatment of 100 uM communic acid to cultured SVEC 4-10 decreased relatively *AT_1 receptor* cDNA expression rather only 30 mM glucose treatment. The *AT_1* receptor inhibition effect of 100 uM communic acid showed similar 100 uM captopril (Fig 18).

As shown in Figure 19, cDNA expression of *eNOS* was regulated with the treatment of communic acid. The treatment of 100 uM communic acid to cultured SVEC 4-10 increased relatively *eNOS* cDNA expression rather only 30 mM glucose treatment. The eNOS induction effect of 100 uM communic acid showed similar 100 uM captopril (Fig 18). The results indicate that communic acid could be an effective ACE inhbitiory, by decreasing the expression level of *AT_1* receptor in SVEC 4-10.

Nitric oxide (NO) is of critical importance as a mediator of vasodilation in blood

vessels. The NO synthesize by eNOS in vascular endothelium results in phosphorylation of several proteins that cause smooth muscle relaxation. The vasodilator actions of NO play a key role in renal control of extracellular fluid homeostasis and is essential for the regulation of blood flow and blood pressure (Yoon et al., 2000) Therefore, communic acid is potentially regulation blood pressure and blood flow through ACE inhibition and NO induction.

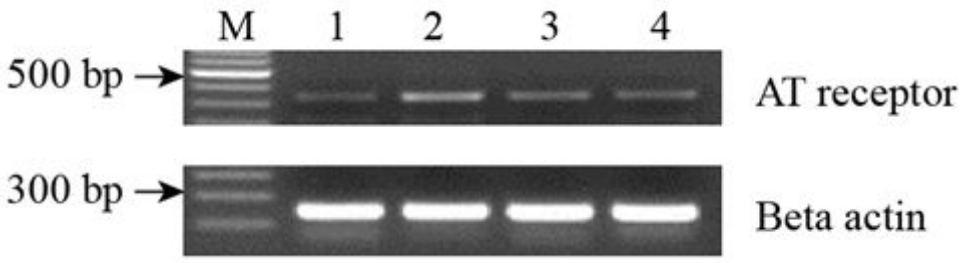

Figure 20. Effect of communic acid on mRNA expression of *angiotensin I receptor* (AT receptor). mRNA expression levels of angiotensin I receptor in the presence of 30 mM glucose. M: 100 bp marker, 1: negative control, 2: 30 mM Glucose, 3: 30 mM Glucose and 100 uM captopril, 4: 30 mM Glucose and 100 uM communic acid. Beta actin was used as an internal control.

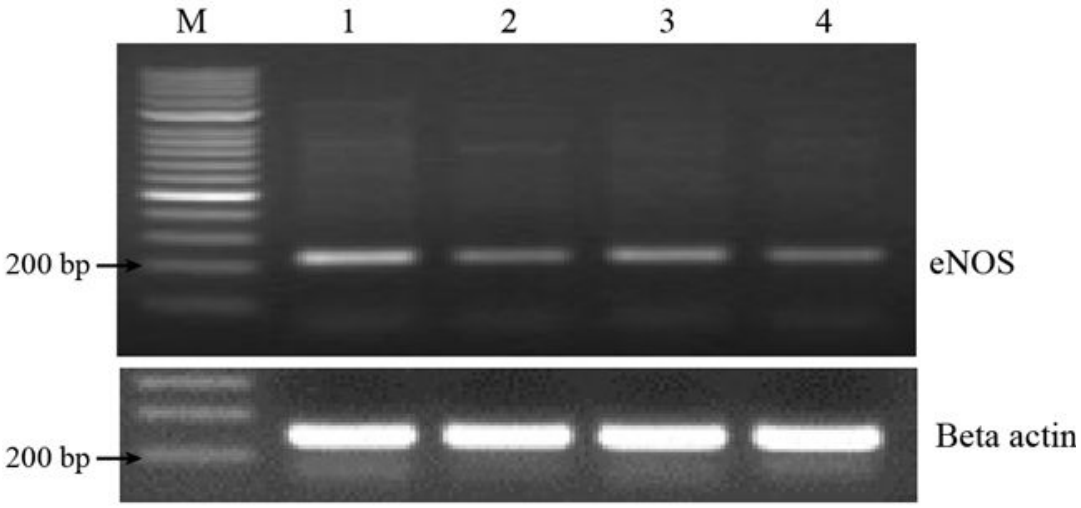

Figure 21. Effect of communic acid on mRNA expression of *eNOS*. mRNA expression levels of angiotensin I receptor in the presence of 30 mM glucose. M: 100 bp marker, 1: negative control, 2: 30 mM Glucose, 3: 30 mM Glucose and 100 uM captopril, 4: 30 mM Glucose and 100 uM communic acid. Beta actin was used as an internal control.

2.3.4 Cytotoxicty determination of dehydroabietic acid and communic acid

Dehydroabietic acid and communic acid were tested for effect on the viability of SVEC 4-10 by the MTT assay. Dehydroabietic acid and communic acid did not show any significant toxicity at the tested concentrations (Fig. 20). As a result, it is identified that dehydroabietic acid and communic acid are safe compounds in period of cellular toxicity. This results could be used as secure ACE inhibitor and as effective antihypertension drug.

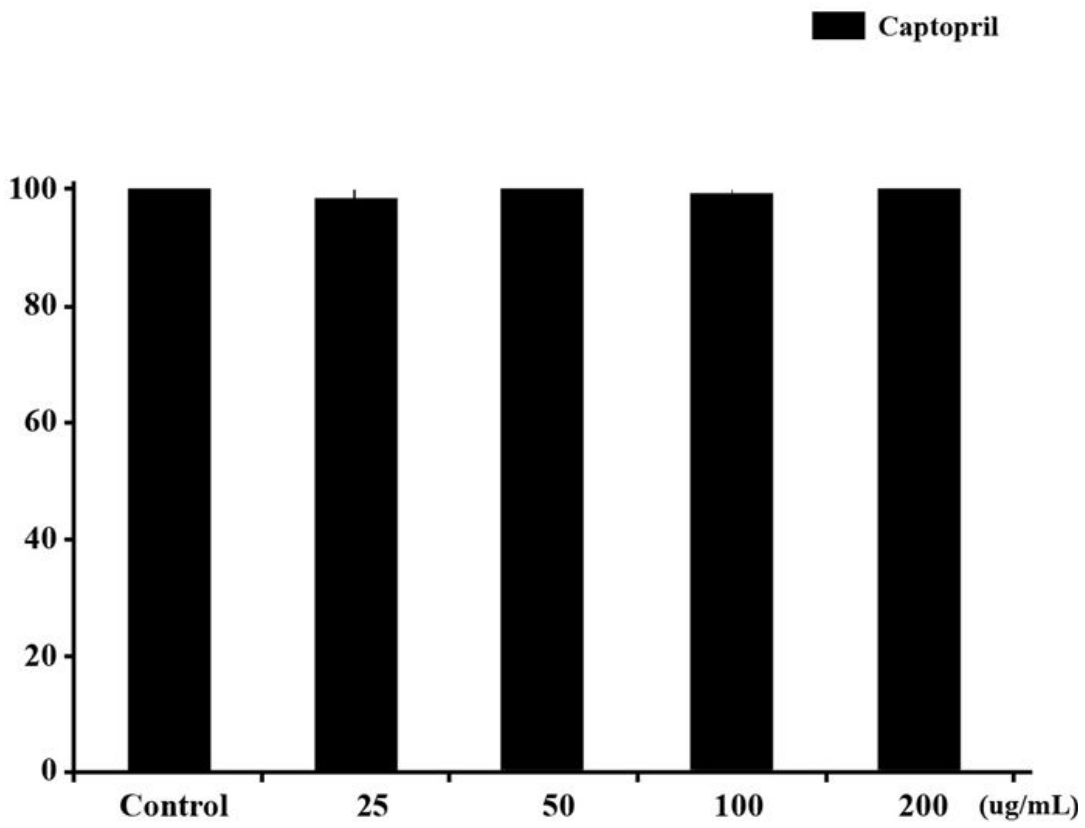

Figure 22. Cytotoxicity of captopril on SVEC. The cell viability was assessed by MTT assay following treatment with different concentration of captopril (25, 50, 100, and 200 ug/mL) for 24 h. Result are means ±SD of three independent expriments.

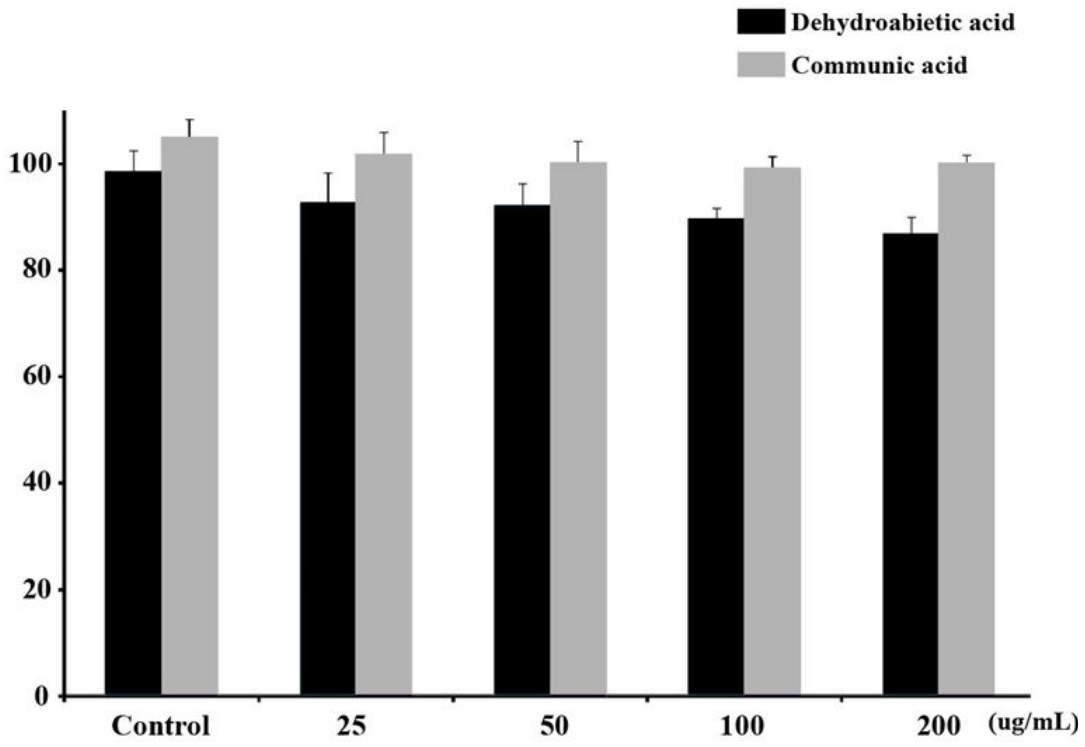

Figure 23. Cytotoxicity of isolated compounds on SVEC. The cell viability was assessed by MTT assay following treatment with different concentration of captopril (25, 50, 100, and 200 ug/mL) for 24 h. Result are means ±SD of three independent expriments.

Chapter 3.
The construction of *Angiotensin converting enzyme* gene

3.1 Introduction

Angiotensin converting enzyme (ACE, dipeptidyl-peptidase A, Kinase II, E.C. 3.4.15.1, DCP1) is a dipeptidyl-carboxy-peptidase that belongs to the M2-metalloprotease family. ACE plays a critical role in blood pressure regulation by converting angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to the vasopressor angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (Acharya et al, 2003, Ehlers et al, 1989). ACE is a important component of the renin angiotensin system (RAS) which controls blood pressure and contractoin of blood vessels (Unger, 2002). For these reasons, antihypertension agents are available important drugs for the treatment of hypertension.

The human ACE has to be divided into two types of somatic ACE (sACE) and testis ACE (tACE). sACE, a type I transmembrane protein, is composed of two homologus catalytic domains called the N and C domains, each including an active catalytic center (Soubrier et al. 1998). tACE contain express in the testes and comprise a single domain with a one active site. tACE is similar the C domain of sACE (Houard et. al., 1998). sACE C domain and tACE active sites cleaves syhthesized peptide Hippuryl-Histin-Leuine (HHL) (Wei et al., 1991 and Natesh et al., 2003) and sACE N domain active site is Angiotensin 1-7 (Asp-Arg-Val-Tyr-Ile-His-Pro), hemoregulatory AcSDKP (acetyl-Ser-Sap-Lys-Pro),

enkephalin precursor Met-Enk-Arg-Phe (Deddish et. al., 1998, Rousseau et al., 1995, and Deddish et. al., 1997).

The antihypertensive agents such as captopril, enalaprilat, lisinopril, ramiprilat, trandolaprilat, perinoprilat, and perinodoprilat binds to the active stie and Zn2+ of ACE (Akif et. al., 2010). To study the binding analysis of two cheomicals (dehydroabietic and communic acids) and ACE protein, ACE cDNA was synthesized in the endothelial cells of house mouse. After ligation with pET-15b, pET-15b-ACE was transformed in DH5α.

3.2 Material and Method

3.2.1 RNA preparation and cDNA synthesis

Total RNA was isolated from house mouse tissures using Hybrid-R (GeneAll) according to the manufacturer's protocol. 500 uL of RiboEx reagent was added and incubated to homogenized tissue samples for 5 min at the room temperature. The sample centrifuge at 12,000 × g for 10 min at 4℃ and transfer the supernatant to a fresh tube. 100 uL of chloroform was added to samples, mixed and store for 2 min at room temperature. The mixture centrifuge at 12,000 × g for 15 min at 4℃ and transfer the aqueous phase to a fresh tube. 50% ethanol added to the sample and transfer to a mini spin column. The samples centrifuged at 10,000 × g for 30 sec at room temperature. 500 uL RBW was added to the mini spin column and was centrifuged at 10,000 × g for 30 sec at room temperature. Then, 500 uL RNW was added the mini spin column and was centrifuged at 10,000 × g for 30 sec at room temperature. Total RNA was harvested and synthesized cDNA. cDNA was synthesized by using the Superscrit First-strand synthesis system, also according to the manufacturer's established protocaol (Invitrogen, Carlsbad, CA). Thereafter, it was reverse-transcripted as following program: 65℃ 10 min, 42℃ 50 min, 70℃ 15 min, and the cDNA product was stored at - 80℃.

3.2.2 Constructs for structure study of ACE and inhibitor binding assay

pET-15b vector including the His·tag and T7 promoter was used for crystal structures of ACE in complex with dehydroabietic acid and communic acid (Fig. 22) (Bernsterin et al., 1989). For generation of expression clones, all inserts were ligated into 5-NdeI and 3-BamHI sites of a pET-15b vector. To prepare insert DNA, ACE form mouse was RT-PCR amplified from cDNA isolated using the GeneAll kit. RT-PCR was performed with n-pfu polymerase (Enzynomics) and the sequecne of the PCR primers were: for the sense strand amplification, a forward primer 5'-TAC ATA TGG AAA ACC TGT ATT TTC AGG GCA TGC TCG ACC CTG GAT TGC-3' named ACE-F7-8 and a reverse primer 5'-TAG GAT CCT CAG GAG TGT CTG AGC TCC ACC TCA GAC CCA A-3' named ACE-R7-9. BamHI and NdeI restriction sites were used for cloning the ACE gene. RT-PCR amplification was performed with Hot-start method and products were examined by gel electrophoresis on 1% agarose gels. Cloning The cloned were confirmed by restriction enzymes. The restriction enzyme was used BamHI and NdeI or BamHI and EcoRI.

3.2.3 DNA sequence

Sequencing reactions are performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) using the ABI BigDye(R) Terminator v3.1 Cycle Sequencing Kit(Applied Biosystems), following the protocols supplied by the manufacturer.

Single-pass sequencing is performed on each template using primer; pET-F1 (GGG TTA TGC TAG TTA TTG CTC AG), ACE-F11 (CCT TGG TGA TGC TTC CAT CA), ACE-F12 (AGT ACT CCA GCA GTG CCT T), ACE-F13 (CTA CGC AGA GAG ACG TGC A), ACE-F14 (CAA CTT GGC ATA GCT TCG TG), and ACE-F15 (CAT GGC TTC CGT GGC ATC TA).

The fluorescent-labeled fragments are purified by the method that Applied Biosystems recommends as it removes the unincorporated terminators and dNTPs. The samples are injected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems).

DNA sequences were translated to protein and aligned using the Gene Runner (5.0.59 Beta) and Clustal-Omega programs.

3.3 Results and discussion

3.3.1 Construction of *Agiotensin converting enzyme* gene

The sequence of house mouse is used NCBI database. Assession number of used ACE sequence is NM-207624.5 and only the mature protein sequence was used. DNA size is 3.831 bp and RT-PCR product is 3,874 bp, including ATG, TAG, BamHI (GGATCC), NdeI (CATATG), and TEV sequence (5'-GAA AAC CTG TAT TTT CAG GGC-3'). TEV sequence is needed to separate ACE protein (Fig. 23).

To cloning mature ACE gene, RT-PCR was carried out using ACE-F7-8 and ACE-R7-9 or ACE-7-10. 1 and 3 were confirmed that the synthesized with ACE full sequence. 2 and 4 were not used to generate the multi-band (Fig. 24). PCR products and pET-15b vector were ligation used to T4 ligase, and pET-15b-ACE was transformed to DH5α.

3.3.2 Confirm transformation on DH5α and sequencing

To confirm the transformation, it was digested by several restriction enzymes, BamHI, EcoRI, and NdeI. BamHI and NdeI will cut insert DNA and vector, and the size of band is predicted 5,696 bp and 3,865 bp, respectively. The 18 colony was confirmed, and it was identified that the five colony clipped to the expected size, 918, 919, 926, 929, and 937 (Fig. 25).

Also, BamHI and EcoRI will cut insert DNA and vector, and the size of band is predicted 6,621 bp and 2,976 bp, respectively. The 18 colony was confirmed, and it was identified that the five colony clipped to the expected size, 918, 919, 926, 929, and 937 (Fig. 26). ACE gene was found that entered in the opposite direction to the pET-15b.

The 918, 919, 926, 929, and 937 colony were confirmed sequence. Sequence alignment used to Clustal Omega (httpp://www2.ebi.uk/clustal omega/). The mouse ACE (NM 207624.5) and pET15b-ACE 937 proteins share 100% amino acid identity. Figure 29 shows a sequence alignment of pET15b-ACE 937 and mouse ACE. The pET15b-ACE 918 and 926 were not correspond with mouse ACE (NM 207624.5).

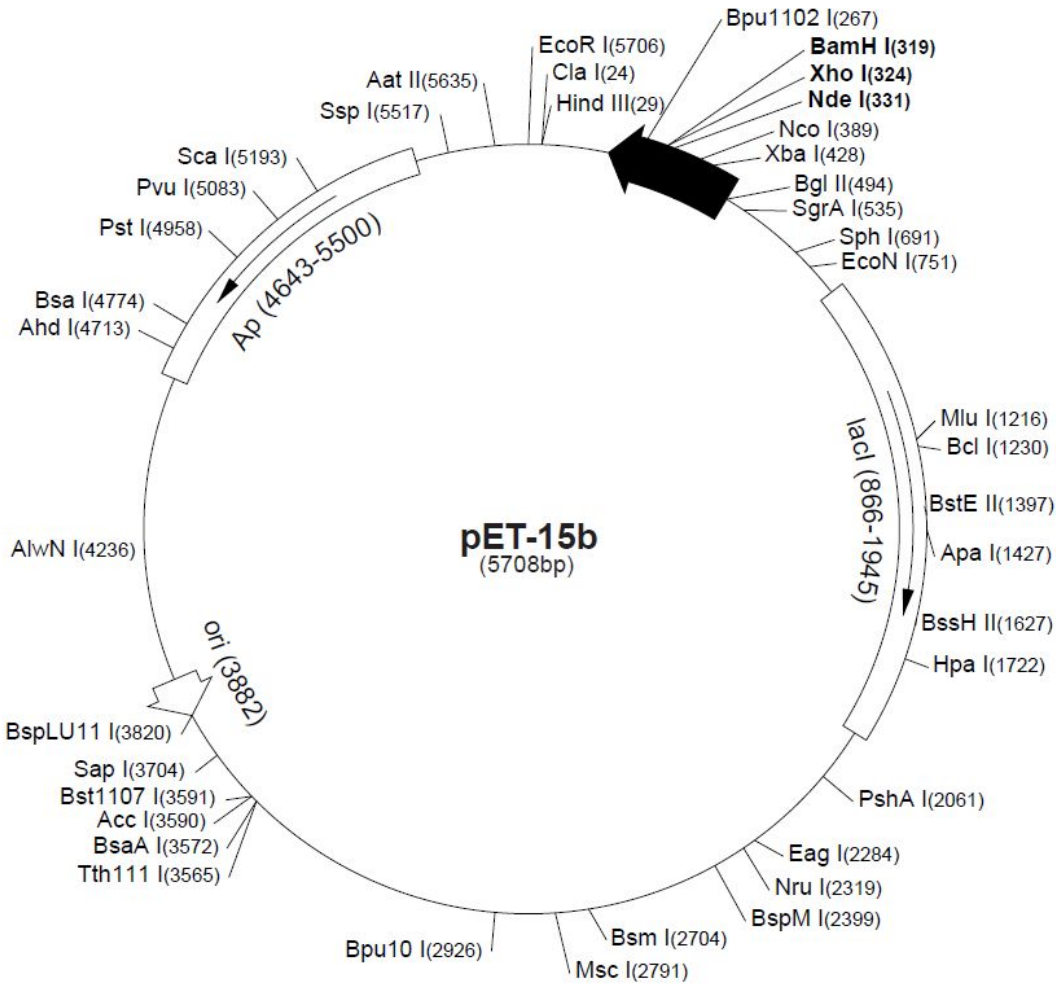

Figure 24. The map of pET-15b vector. This vector is composed with N-terminal His·Tag, T7 promoter, lac operator gene, thrombin site, and three cloning sites. T7 expression region is particularly reversed on the circular map. Cloning and protein expression are selection in DH5α and BL21(DE3), respectively.

CTCGACCCTGGATTGCAGCCGGGCAACTTTTCCCCGGACGAGGCAGGGGCG
CAGCTTTTCGCTGAAAGCTATAACTCGAGTGCCGAGGTGGTGATGTTCCAGA
GCACCGTGGCCAGTTGGGCGCACGACACCAACATCACGGAGGAGAACGCGC
GACGCCAGGAGGAAGCGGCCCTGGTCAGCCAGGAGTTTGCAGAGGTCTGGG
GCAAGAAGGCCAAGGAGTTGTATGAGTCCATTTGGCAGAACTTTACTGACTCA
AAGCTGCGAAGGATCATCGGATCTATTCGGACCCTAGGACCTGCCAATCTGC
CCCTGGCCCAGCGGCAGCAGTACAACTCTCTGCTAAGCAACATGAGCAGAAT
CTACTCCACTGGCAAGGTCTGCTTCCCCAACAAGACTGCCACCTGCTGGTCC
CTGGACCCAGAGCTCACCAACATCCTGGCTTCCTCACGAAGCTATGCCAAGTT
GTTGTTTGCCTGGGAGGGCTGGCATGATGCTGTGGGTATCCCACTGAAACCC
CTCTATCAAGACTTTACTGCCATCAGTAACGAAGCCTACAGACAAGACGACTT
CTCAGACACAGGAGCCTTCTGGCGCTCCTGGTATGAGTCCCCCTCCTTTGAG
GAGAGTCTGGAACATATCTACCACCAACTAGAGCCCCTCTACCTGAACCTCCA
TGCCTACGTCCGCCGCGCACTGCACCGCCGCTATGGGGACAAATACGTCAAT
CTCAGGGGGCCTATTCCTGCCCATTTGCTGGGAGACATGTGGGCCCAGAGCT
GGGAGAACATCTACGACATGGTAGTGCCTTTCCCAGACAAACCCAACCTCGA
TGTCACCAGTACAATGGTACAGAAGGGCTGGAACGCCACACACATGTTCCGG
GTATCAGAGGAATTCTTCACCTCGCTGGGGCTCTCACCCATGCCTCCTGAGTT
CTGGGCGGAGTCAATGCTGGAGAAACCAACGGATGGACGGGAGGTGGTGTG
CCACGCCTCTGCCTGGGACTTCTACAACCGGAAGGACTTCCGGATTAAGCAA
TGCACACGGGTCACGATGGAACAGCTGGCCACAGTACACCACGAGATGGGC
CACGTGCAGTACTACCTCCAGTACAAGGACCTGCACGTCTCTCTGCGTAGAG
GTGCCAACCCTGGCTTCCATGAGGCCATTGGGGATGTGCTTGCACTCTCCGT
CTCTACCCCTGCACATCTGCACAAAATCGGCCTACTGGACCATGTTACCAATG
ACATAGAGAGTGACATCAATTACCTGCTAAAGATGGCCCTAGAGAAAATCGCC
TTCTTGCCCTTTGGCTACCTGGTGGACCAGTGGCGTTGGGGGGTCTTCAGTG
GACGGACCCCACCCTCTCGCTACAACTTCGACTGGTGGTATCTTCGAACCAA
GTATCAGGGGATCTGCCCACCAGTTGCCCGGAATGAAACCCATTTTGATGCT

GGAGCCAAGTTTCACATCCCAAACGTGACACCGTACATCAGGTACTTCGTGA
GCTTTGTGCTGCAGTTCCAGTTCCATCAAGCACTGTGCAAGGAGGCAGGCCA
CCAGGGCCCACTACACCAGTGTGACATCTACCAGTCCGCCCAGGCGGGGGC
CAAGCTCAAGCAGGTGTTGCAGGCTGGCTGCTCCAGGCCCTGGCAGGAGGT
ACTGAAGGACCTGGTAGGCTCAGATGCCCTGGATGCCAAGGCACTGCTGGA
GTACTTCCAACCGGTCAGCCAGTGGCTGGAAGAGCAGAATCAGCGGAATGGC
GAAGTCCTAGGCTGGCCAGAGAATCAGTGGCGTCCACCGTTACCCGACAACT
ATCCAGAGGGCATTGACCTAGAGACTGATGAAGCCAAGGCTGACAGGTTCGT
GGAAGAGTATGACCGGACAGCCCAAGTGTTGTTGAACGAGTACGCAGAGGCC
AACTGGCAATATAACACCAACATTACCATAGAGGGCAGCAAGATCCTGCTTGA
GAAAAGCACGGAGGTATCCAATCACACCCTGAAATATGGCACCCGGGCCAAG
ACATTTGATGTGAGCAACTTTCAAAACTCTTCCATCAAGCGGATCATAAAGAAG
CTTCAGAACCTGGACCGGGCAGTGCTGCCTCCCAAGGAATTAGAAGAGTACA
ACCAGATCCTGCTAGACATGGAGACAACTTACAGCTTATCCAACATTTGCTAC
ACAAATGGCACTTGTATGCCCCTGGAACCTGATCTAACAAACATGATGGCCAC
ATCCCGGAAATATGAAGAATTGCTATGGGCATGGAAGAGCTGGAGAGACAAG
GTGGGGAGAGCCATCCTTCCTTTTTTCCCAAAGTATGTGGAGTTCTCCAACAA
GATTGCCAAGCTCAATGGCTACACGGATGCAGGGGATTCATGGAGATCCTTA
TACGAGTCTGACAACCTGGAGCAAGACCTGGAAAAACTGTACCAGGAGCTGC
AGCCACTCTACCTGAACCTGCATGCCTATGTGCGTCGTTCCCTGCACCGCCA
CTATGGGTCCGAGTACATCAACCTGGATGGCCCCATTCCTGCCCATCTGCTA
GGGAACATGTGGGCGCAGACCTGGTCCAACATCTATGATTTGGTGGCGCCCT
TCCCTTCCGCCCCCAATATAGATGCCACGGAAGCCATGATAAAGCAGGGATG
GACACCCAGAAGGATATTTAAGGAAGCTGACAATTTCTTTACCTCCCTGGGGC
TGTTACCTGTGCCCCCTGAGTTCTGGAACAAGTCGATGTTAGAGAAGCCCACC
GATGGAAGGGAGGTGGTGTGCCATCCCTCAGCCTGGGACTTCTACAACGGCA
AGGACTTCAGGATCAAGCAGTGTACCTCTGTGAACATGGAGGACTTGGTGAT
AGCGCACCACGAAATGGGCCACATCCAGTATTTCATGCAGTACAAAGACTTAC

CCGTGACTTTCCGGGAGGGTGCCAACCCTGGTTTTCATGAAGCTATTGGAGA
TATAATGGCTCTCTCAGTGTCTACCCCCAAGCATCTATACAGTCTCAACCTGCT
TAGCACTGAGGGCAGTGGCTACGAGTATGACATCAACTTTCTAATGAAGATGG
CCCTCGACAAGATCGCCTTTATCCCCTTCAGCTACCTCATCGACCAGTGGCGC
TGGAGGGTCTTTGATGGAAGCATCACCAAGGAGAACTATAACCAGGAGTGGT
GGAGCCTCAGGCTGAAGTATCAGGGTCTGTGCCCCCCAGTGCCAAGATCCCA
AGGTGACTTTGACCCAGGGTCCAAGTTCCACGTTCCTGCGAACGTGCCATAC
GTCAGGTACTTTGTCAGCTTCATCATCCAGTTCCAGTTCCACGAGGCGCTGTG
TCGCGCAGCCGGGCACACGGGTCCCCTGCACAAGTGTGACATCTACCAATCC
AAGGAAGCAGGGAAGCTCCTGGCGGATGCCATGAAGCTGGGCTACAGTAAG
CCGTGGCCAGAGGCCATGAAGCTGATCACAGGCCAGCCTAACATGTCAGCCT
CCGCCATGATGAATTACTTCAAGCCACTGACAGAATGGCTCGTCACCGAGAA
CAGGAGACATGGAGAGACACTGGGCTGGCCGGAGTACAACTGGGCGCCAAA
CACCGCTCGCGCAGAAGGCTCCACCGCAGAGTCCAACCGCGTCAATTTCCTG
GGCCTGTACCTGGAGCCACAGCAGGCCCGCGTGGGCCAGTGGGTGCTGCTC
TTCCTGGGCGTCGCCCTGCTCGTGGCCACCGTGGGTCTCGCCCATCGGCTCT
ACAACATCCGTAACCATCACAGCCTCCGCCGGCCCCACCGTGGGCCCCAGTT
TGGGTCTGAGGTGGAGCTCAGACAC

Figure 25. DNA Sequence of ACE mature protein. DNA size is 3.831 bp. Assession number is NM-207624.5. mature protein start is 140 nucleotide and stop is 3,973 nucleotide.

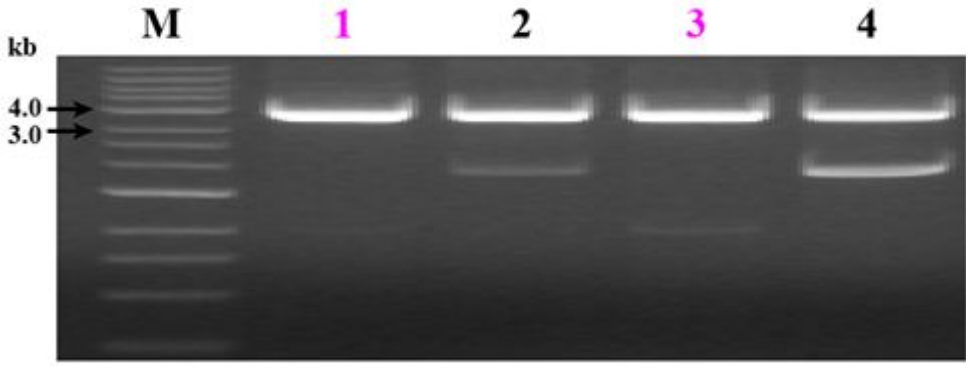

Figure 26. RT-PCR result of ACE. ACE DNA was amplified by forward and reverse primers. 1 and 3 were used ACE-F7-8 and ACE-R7-9. 2 and 4 were used ACE-F7-8 and ACE-R7-10. M is 1,000 bp marker.

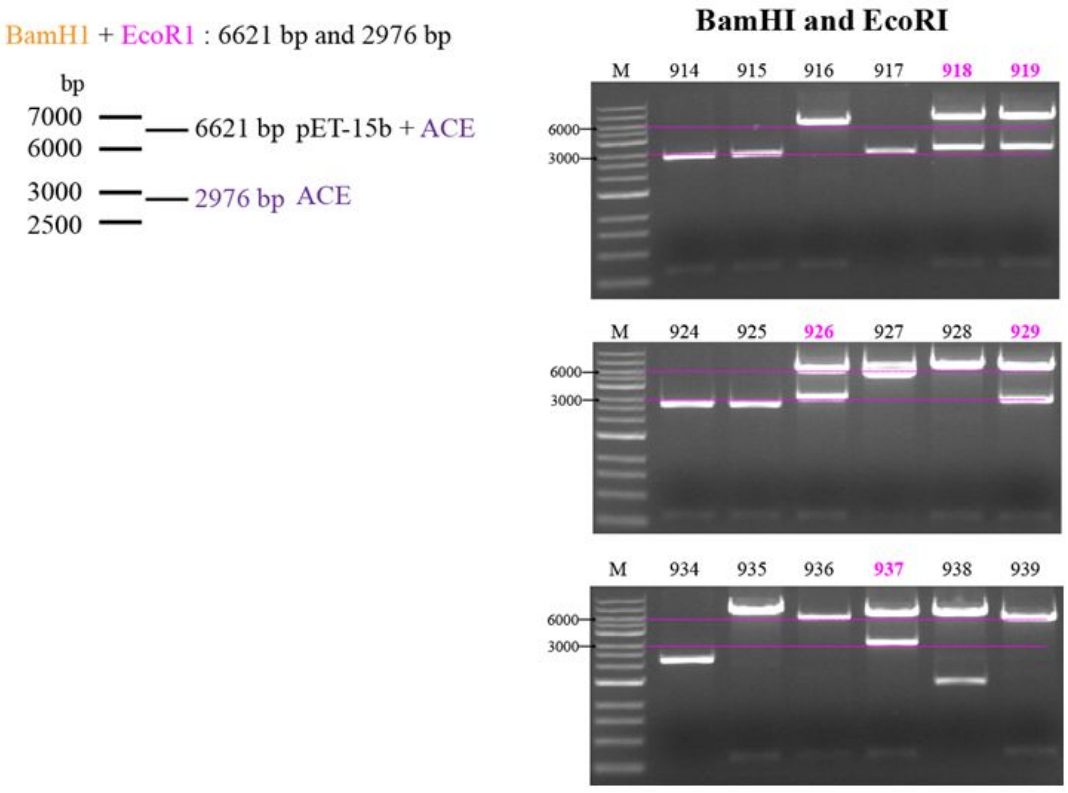

Figure 27. The expected band and a real band by BamHI and EcoRI. Left image is the expected band by BamHI and EcoRI. Right image is a real band by BamHI and EcoRI.

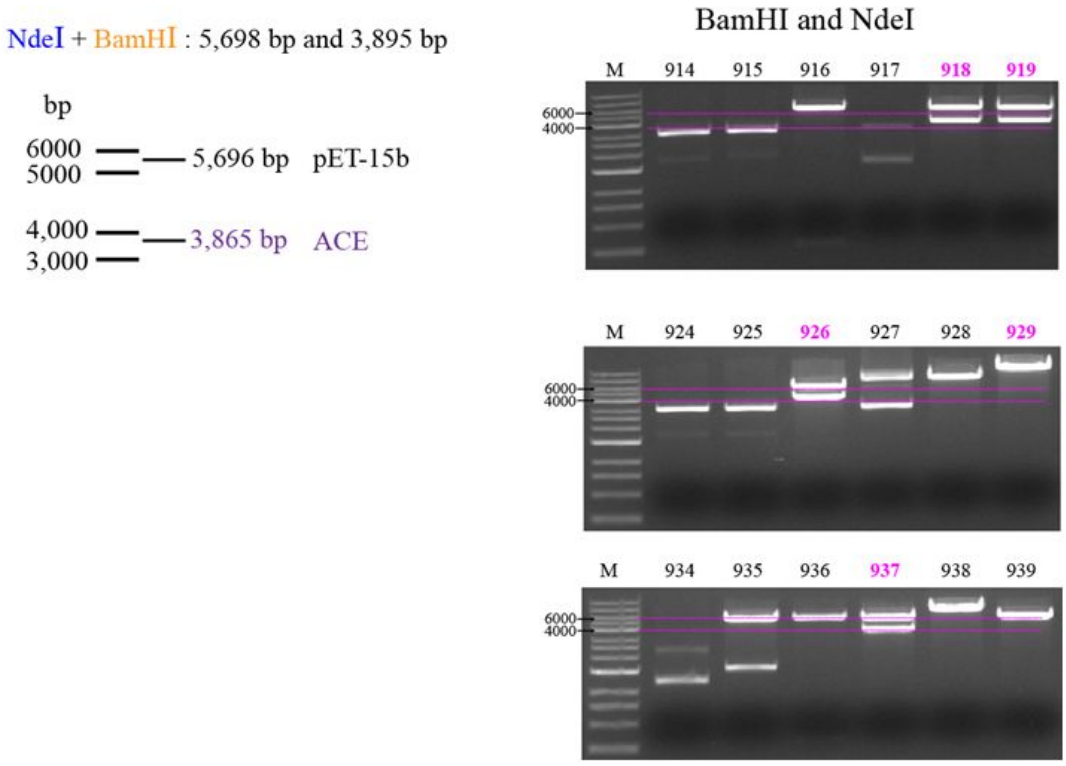

Figure 28. The expected band and a actual band by BamHI and NdeI. Left image is the expected band by BamHI and NdeI. Right image is a real band by BamHI and NdeI.

Figure 29. The map of construction *ACE* gene. The construct map for ACE in vector pET-15b.

```
pET15b-ACE      HMENLYFQGMLDPGLQPGNFSPDEAGAQLFAESYNSSAEVVMFQSTVASWAHDTNITEEN
Mouse ACE                 LDPGLQPGNFSPDEAGAQLFAESYNSSAEVVMFQSTVASWAHDTNITEEN

pET15b-ACE      ARRQEEAALVSQEFAEVWGKKAKELYESIWQNFTDSKLRRIIGSIRTLGPANLPLAQRQQ
Mouse ACE       ARRQEEAALVSQEFAEVWGKKAKELYESIWQNFTDSKLRRIIGSIRTLGPANLPLAQRQQ

pET15b-ACE      YNSLLSNMSRIYSTGKVCFPNKTATCWSLDPELTNILASSRSYAKLLFAWEGWHDAVGIP
Mouse ACE       YNSLLSNMSRIYSTGKVCFPNKTATCWSLDPELTNILASSRSYAKLLFAWEGWHDAVGIP

pET15b-ACE      LKPLYQDFTAISNEAYRQDDFSDTGAFWRSWYESPSFEESLEHIYHQLEPLYLNLHAYVR
Mouse ACE       LKPLYQDFTAISNEAYRQDDFSDTGAFWRSWYESPSFEESLEHIYHQLEPLYLNLHAYVR

pET15b-ACE      RALHRRYGDKYVNLRGPIPAHLLGDMWAQSWENIYDMVVPFPDKPNLDVTSTMVQKGWNA
Mouse ACE       RALHRRYGDKYVNLRGPIPAHLLGDMWAQSWENIYDMVVPFPDKPNLDVTSTMVQKGWNA

pET15b-ACE      THMFRVSEEFFTSLGLSPMPPEFWAESMLEKPTDGREVVCHASAWDFYNRKDFRIKQCTR
Mouse ACE       THMFRVSEEFFTSLGLSPMPPEFWAESMLEKPTDGREVVCHASAWDFYNRKDFRIKQCTR

pET15b-ACE      VTMEQLATVHHEMGHVQYYLQYKDLHVSLRRGANPGFHEAIGDVLALSVSTPAHLHKIGL
Mouse ACE       VTMEQLATVHHEMGHVQYYLQYKDLHVSLRRGANPGFHEAIGDVLALSVSTPAHLHKIGL

pET15b-ACE      LDHVTNDIESDINYLLKMALEKIAFLPFGYLVDQWRWGVFSGRTPPSRYNFDWWYLRTKY
Mouse ACE       LDHVTNDIESDINYLLKMALEKIAFLPFGYLVDQWRWGVFSGRTPPSRYNFDWWYLRTKY

pET15b-ACE      QGICPPVARNETHFDAGAKFHIPNVTPYIRYFVSFVLQFQFHQALCKEAGHQGPLHQCDI
Mouse ACE       QGICPPVARNETHFDAGAKFHIPNVTPYIRYFVSFVLQFQFHQALCKEAGHQGPLHQCDI

pET15b-ACE      YQSAQAGAKLKQVLQAGCSRPWQEVLKDLVGSDALDAKALLEYFQPVSQWLEEQNQRNGE
Mouse ACE       YQSAQAGAKLKQVLQAGCSRPWQEVLKDLVGSDALDAKALLEYFQPVSQWLEEQNQRNGE

pET15b-ACE      VLGWPENQWRPPLPDNYPEGIDLETDEAKADRFVEEYDRTAQVLLNEYAEANWQYNTNIT
Mouse ACE       VLGWPENQWRPPLPDNYPEGIDLETDEAKADRFVEEYDRTAQVLLNEYAEANWQYNTNIT
```

Figure 30. Sequence alignment analysis of pET15b-ACE and mouse ACE protein sequences. Each protein indicated the left of the alignment. The enzyme restriction site at each end of pET15b-ACE is red letter. The amino acid HM are NdeI site and GS are BamH1 site. The amino acid ENLYFQGM are TEV site.

pET15b-ACE	IEGSKILLEKSTEVSNHTLKYGTRAKTFDVSNFQNSSIKRIIKKLQNLDRAVLPPKELEE
Mouse ACE	IEGSKILLEKSTEVSNHTLKYGTRAKTFDVSNFQNSSIKRIIKKLQNLDRAVLPPKELEE
pET15b-ACE	YNQILLDMETTYSLSNICYTNGTCMPLEPDLTNMMATSRKYEELLWAWKSWRDKVGRAIL
Mouse ACE	YNQILLDMETTYSLSNICYTNGTCMPLEPDLTNMMATSRKYEELLWAWKSWRDKVGRAIL
pET15b-ACE	PFFPKYVEFSNKIAKLNGYTDAGDSWRSLYESDNLEQDLEKLYQELQPLYLNLHAYVRRS
Mouse ACE	PFFPKYVEFSNKIAKLNGYTDAGDSWRSLYESDNLEQDLEKLYQELQPLYLNLHAYVRRS
pET15b-ACE	LHRHYGSEYINLDGPIPAHLLGNMWAQTWSNIYDLVAPFPSAPNIDATEAMIKQGWTPRR
Mouse ACE	LHRHYGSEYINLDGPIPAHLLGNMWAQTWSNIYDLVAPFPSAPNIDATEAMIKQGWTPRR
pET15b-ACE	IFKEADNFFTSLGLLPVPPEFWNKSMLEKPTDGREVVCHPSAWDFYNGKDFRIKQCTSVN
Mouse ACE	IFKEADNFFTSLGLLPVPPEFWNKSMLEKPTDGREVVCHPSAWDFYNGKDFRIKQCTSVN
pET15b-ACE	MEDLVIAHHEMGHIQYFMQYKDLPVTFREGANPGFHEAIGDIMALSVSTPKHLYSLNLLS
Mouse ACE	MEDLVIAHHEMGHIQYFMQYKDLPVTFREGANPGFHEAIGDIMALSVSTPKHLYSLNLLS
pET15b-ACE	TEGSGYEYDINFLMKMALDKIAFIPFSYLIDQWRWRVFDGSITKENYNQEWWSLRLKYQG
Mouse ACE	TEGSGYEYDINFLMKMALDKIAFIPFSYLIDQWRWRVFDGSITKENYNQEWWSLRLKYQG
pET15b-ACE	LCPPVPRSQGDFDPGSKFHVPANVPYVRYFVSFIIQFQFHEALCRAAGHTGPLHKCDIYQ
Mouse ACE	LCPPVPRSQGDFDPGSKFHVPANVPYVRYFVSFIIQFQFHEALCRAAGHTGPLHKCDIYQ
pET15b-ACE	SKEAGKLLADAMKLGYSKPWPEAMKLITGQPNMSASAMMNYFKPLTEWLVTENRRHGETL
Mouse ACE	SKEAGKLLADAMKLGYSKPWPEAMKLITGQPNMSASAMMNYFKPLTEWLVTENRRHGETL
pET15b-ACE	GWPEYNWAPNTARAEGSTAESNRVNFLGLYLEPQQARVGQWVLLFLGVALLVATVGLAHR
Mouse ACE	GWPEYNWAPNTARAEGSTAESNRVNFLGLYLEPQQARVGQWVLLFLGVALLVATVGLAHR
pET15b-ACE	LYNIRNHHSLRRPHRGPQFGSEVELRHSGS
Mouse ACE	LYNIRNHHSLRRPHRGPQFGSEVELRHS

Figure 29. Alignment analysis of pET15b-ACE and mouse ACE amino acid sequences (continued on previous page).

REFERENCES

Acharya, K. R., E. D. Sturrock, et al. (2003). ACE revisited: a new target for structure-based drug design. Nature Reviews Drug Discovery 2(11): 891-902.

Actis-Goretta, L., Ottaviani, J. I., Keen, C. L., Fraga, C. G., Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins. FEBS Letters. 555: 597-600.

Ahn, M. Y., Hahn, B.S., Ryu, K.S., Kim, J.W., Kim, I., Kim, Y.S. (2003). Purification and characterization of a serine protease with fibrinolytic activity from the dung beetles, Catharsius molossus. Thromb Res. 112(5): 339-347.

Ajjan, R. A., Standeven, K.F., Khanbhai, M., Phoenix, F., Gersh, K.C., Weisel, J.W., Kearney, M.T., Ariëns, R.A.S., Grant, P.J. (2009). Effects of aspirin on clot structure and fibrinolysis using a novel in vitro cellular system. Arterioscler. Thromb. Vasc. Biol. 29(5): 712-717.

Antovic, A., Perneby, C., Ekman, G.J., Wallen, H.N., Hjemdahl, P., Blombäck, M., He, S. (2005). Marked increase of fibrin gel permeability with very low dose ASA treatment. Thromb Res. 116(6): 509-517.

Atlas, S. A., The renin-angiotensin aldosterone system: pahtophysiological role and pharmacologic inhibition. J Manag Care Pham. 13(8): 9-20

Barrero, A. F., Oltra, J.E., Poyatos, J.A. (1996). Acidic metabolites from Phycomyces blakesleeanus. Phytochemistry 42(5): 1427-1433.

Bernstein, K., B. Martin, et al. (1989). Mouse angiotensin-converting enzyme is a protein composed of two homologous domains. Journal of Biological Chemistry 264(20): 11945-11951.

Bertelli, A. A. E., Mannari, C., Santi, S., Filippi, C., Migliori, M., Giovannini, L. (2008). Immunomodulatory activity of shikimic acid and quercitin in comparison with oseltamivir (Tamiflu) in an “in vitro” model. J. Med. Virol. 80(4): 741-745.

Bjornsson, T. D., Schneider, D.E., Berger, H. (1989). Aspirin acetylates fibrinogen and enhances fibrinolysis. Fibrinolytic effect is independent of changes in plasminogen activator levels. J. Pharmacol. Exp. Ther. 250(1): 154-161.

Borazan, A. A., Bozan, B. (2013). The influence of pectolytic enzyme addition and prefermentative mash heating during the winemaking process on the phenolic composition of Okuzgozu red wine. Food Chem. 138(1): 389-395.

Caboun, V. (2006). Tree-tree allelopathic interactions in middle European Forests. Allelopathy J. 17: 17-31.

Carr, M. E., Hermans, J. (1978). Size and density of fibrin fibers from turbidity. Macromolecules 11(1): 46-50.

Cesarman-Maus, G. and K. A. Hajjar (2005). Molecular mechanisms of fibrinolysis. Br. J. Haematal 129(3): 307-321.

Choi, E. M. (2007). Antinociceptive and antiinflammatory activities of pine (Pinus densiflora) pollen extract. Phytother. Res. 21(5): 471-475.

Choi, N. S., Yoo, K.H., Hahm, J.H., Yoon, K.S., Chang, K.T., Hyun, B.H., Maeng, P.J., 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(1): 72-79.

Chou, T. H., Ding, H.Y., Lin, R.J., Liang, J.Y., Liang, C.H. (2010). Inhibition of melanogenesis and oxidation by protocatechuic acid from Origanum vulgare (oregano). J. Nat. Prod. 73(11): 1767-1774.

Collet, J. P., Allali, Y., Lesty, C., Tanguy, M.L., Silvain, J., Ankri, A., Blanchet, B.,

Dumaine, R., Gianetti, J., Payot, L. (2006). Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. Arterioscl. Thromb. Vasc. Biol. 26(11): 2567-2573.

Colombo, S. L., Andreo, C.S., Chollet, R. (1998). The interaction of shikimic acid and protein phosphorylation with pep carboxylase from the C4 dicot Amaranthus viridis. Phytochemistry 48(1): 55-59.

Deddish, P. A., B. Marcic, et al. (1998). N-Domain–Specific Substrate and C-Domain Inhibitors of Angiotensin-Converting Enzyme Angiotensin-(1–7) and Keto-ACE. Hypertension 31(4): 912-917.

Ehlers, M. R. and J. F. Riordan (1989). Angiotensin-converting enzyme: new concepts concerning its biological role. Biochemistry 28(13): 5311-5318.

Enrich, L. B., Scheuermann, M.L., Mohadjer, A., Matthias, K.R., Eller, C.F., Newman, M.S., Fujinaka, M., Poon, T. (2008). Liquidambar styraciflua: a renewable source of shikimic acid. Tetrahedron Lett. 49(16): 2503-2505.

Fatah, K., Beving, H., Albåge, A., Ivert, T., Blombäck, M. (1996). Acetylsalicylic acid may protect the patient by increasing fibrin gel porosity. Is withdrawing of treatment harmful to the patient? Eur. Heart J. 17(9): 1362-1366.

Feio, S., B. Gigante, et al. (1997). Method on multiwell plates for the evaluation of the antimicrobial activity of resin acid derivatives. Journal of Microbiological Methods 28(3): 201-206.

Ferrario, C. M., (2010) Addressing the theoretical and clinical advantages of combination theraphy with inhibitors of the renin-angiotensin-aldosterone system: Antihypertensive effects and benefits beyond BP control, Life sciences 86: 289-299.

Fersht, A. (1999). Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding, Macmillan.

Francis, C. W., Marder, V.J., Barlow, G.H. (1980). Plasmic degradation of crosslinked fibrin: characterization of new macromolecular soluble complexes and a model of their structure. J. Clin. Invest. 66(5): 1033-1043.

Ginjom, I., D'Archy, B., Caffin, N., Gidley, M. (2011). Phenolic compound profiles in selected Queensland red wines at all stages of the wine-making process. Food Chem. 125(3): 823-834.

Henschen, A., McDonagh, J., Ed. (1986). Fibrinogen, fibrin and factor XIII, in: Zwaal, R. F. A.; Hemker, H. C. (Eds), Blood Coagulation, Elsevier Science, Amsterdam.

Herrmann, K. M. (1995). The shikimate pathway: early steps in the biosynthesis of aromatic compounds. Plant Cell 7(7): 907-919.

Hiroki, O., Naota, T., Taku, M.A., Masaru, W., Smith, R.L. (2009). Rapid separation of shikimic acid from Chinese star anise (Illicium verum Hook. f.) with hot water extraction. Sep. Purif. Technol. 69(1): 102-108.

Houard, X., T. A. Williams, et al. (1998). The Drosophila melanogaster-related angiotensin-I-converting enzymes Acer and Ance. European journal of biochemistry 257(3): 599-606.

Ivanova, V., Vojnoski, B., Stefova, M. (2011). Effect of the winemaking practices and aging on phenolic content of Smederevka and Chardonnay wines. Food Bioprocess Technology 4(8): 1512-1518.

Jeong, Y. K., Park, J.U., Baek, H., Park, S.H., Kong, I.S., Kim, D.W., Joo, W.H. (2001). Purification and biochemical characterization of a fibrinolytic enzyme from Bacillus subtilis BK-17. World J. Microbiol. Biotechnol. 17(1): 89-92.

Jia, Y. H., Jin, Y., Lü, Q.M., Li, D.S., Wang, W.Y., Xiong, Y.L. (2003). Jerdonase, a Novel Serine Protease with Kinin-releasing and Fibrinogenolytic Activity from Trimeresurus jerdonii Venom. Acta Biochim. Biophys. Sinica 35: 689-694.

Jiang, S., Singh, G., Boam, D.J., Coggins, J.R. (1999). Synthesis of 3-deoxy-3, 3-difluoroshikimic acid and its 4-epimer from quinic acid. Tetrahedron: Asymmetry 10(21): 4087-4090.

Johansson, L., Lindskog, A., Silfversparre, G., Cimander, C., Nielsen, K.F., Lidén, G. (2005). Shikimic acid production by a modified strain of E. coli (W3110. shik1) under phosphate limited and carbon limited conditions. Biotechnol. Bioeng. 92(5): 541-552.

Jung, M. J., Chung, H.Y., Choi, J.H., Choi, J.S. (2003). Antioxidant principles from the needles of red pine, Pinus densiflora. Phytother. Res. 17(9): 1064-1068.

Kim, K. Y., Chung, H.J (2000). Flavor Compounds of Pine Sprout Tea and Pine Needle Tea. J. Agric. Food Chem. 48: 1269-1272.

Kinouchi, Y., H. Ohtsu, et al. (2000). Potential Antitumor-Promoting Diterpenoids from the Stem Bark of Picea g lehni. Journal of natural products 63(6): 817-820.

Krege, J. H., S. W. John, et al. (1995). Male female differences in fertility and blood pressure in ACE-deficient mice. Nature 375(6527): 146-148.

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

Lin, H. H., Chen, J.H., Huang, C.C., Wang, C.J. (2007). Apoptotic effect of 3, 4-dihydroxybenzoic acid on human gastric carcinoma cells involving JNK/p38 MAPK signaling activation. Int. J. Cancer 120(11): 2306-2316.

Lin, W. L., Hsieh, Y.J., Chou, F.P., Wang, C.J., Cheng, M.T., Tseng, T.H. (2003). Hibiscus protocatechuic acid inhibits lipopolysaccharide-induced rat hepatic damage. Arch. Toxicol. 77(1): 42-47.

Lord, S. T. (2011). Molecular mechanisms affecting fibrin structure and stability. Arterioscler. Thromb. Vasc. Biol. 31(3): 494-499.

Martin, D., D. Tholl, et al. (2002). Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant physiology 129(3): 1003-1018.

McKee, P. A., Rogers, L.A., Marler, E.H., Robert L. (1966). The subunit polypeptides of human fibrinogen. Arch. Biochem. Biophys. 116: 271-279.

Mihara, H., Sumi, H., Yoneta, T., Mizumoto, H., Ikeda, R., Seiki, M., Maruyama, M. (1990). A novel fibrinolytic enzyme extracted from the earthworm, Lumbricus rubellus. Jpn. J. Physiol. 41(3): 461-472.

Natesh, R., S. L. Schwager, et al. (2003). Crystal structure of the human angiotensin-converting enzyme–lisinopril complex. Nature 421(6922): 551-554.

Park, G. Y., Paudyal, D.P., Park, Y.M.,Lee, C.S.,Hwang, I.D., Tripathi, G.R., Cheong, H.S. (2008). Effects of pine needle extracts on plasma cholesterol, fibrinolysis and gastrointestinal motility. Biotechnol. Bioprocess Eng. 13(2): 262-268.

Park, G. Y., Paudyal, D.P., Hwang, I.D., Tripathi, G.R., Yang, Y.K., Cheong, H.S. (2008). Production of fermented needle extracts from red pine and their functional characterization. Biotechnol. Bioprocess Eng. 13(2): 256-261.

Patrono, C., García Rodríguez, L.A., Landolfi, R., Baigent, C. (2005). Low-dose aspirin for the prevention of atherothrombosis. N Engl J Med. 353(22): 2373-2383.

Pizzo, S. V., Schwartz, M.L., Hill, R.L., McKee, P.A. (1972). The effect of plasmin on the subunit structure of human fibrinogen. J. Biol. Chem. 247(3): 636-645.

Pizzo, S. V., Schwartz, M.L., Hill, R.L., McKee, P. A. (1973). The effect of plasmin on the subunit structure of human fibrin. J. Biol. Chem. 248(13): 4574-4583.

Pizzo, S. V., M. L. Schwartz, et al. (1972). The effect of plasmin on the subunit structure of human fibrinogen. Journal of Biological Chemistry 247(3): 636-645.

Prinz, S., U. Müllner, et al. (2002). Oxidation products of abietic acid and its methyl ester. Journal of natural products 65(11): 1530-1534.

Shui, G., Bao, Y.M., Bo, J., An L. (2006). Protective effect of protocatechuic acid from Alpinia oxyphylla on hydrogen peroxide-induced oxidative PC12 cell death. J. Eur. Pharmacol. 538(1): 73-79.

Shui, G., Dan, G., Liua, T.Q., Maa, X.H., Cuib, Z.F. (2009). Protocatechuic acid promotes cell proliferation and reduces basal apoptosis in cultured neural stem cells. Toxicol. In Vitro. 23(2): 201-208.

Shulman, S., Katz, S., Ferry, J.D. (1953). The conversion of fibrinogen to fibrin. XII. Influence of pH, ionic strength and hexamethylene glycol concentration on the polymerization of fibrinogen. Arch. Biochem. Biophys. 42(2): 245-256.

Soubrier, F., F. Alhenc-Gelas, et al. (1988). Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. Proceedings of the National Academy of Sciences 85(24): 9386-9390.

Sui, R. (2008). Separation of shikimic acid from pine needles. Chem. Eng. Technol. 31(3): 469-473.

Sumi, H., Nakajima, N., Mihara, H. (1992). Fibrinolysis relating substances in marine creatures. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 102(1): 163-167.

Sydenham, E., A. D. Dangour, et al. (2012). Omega 3 fatty acid for the prevention of cognitive decline and dementia. Sao Paulo Medical Journal 130(6): 419-419.

Tagat, J. R., D. V. Nazareno, et al. (1994). Synthesis and anti-herpes activity of some a-ring functionalized dehydroabietane derivatives. Bioorganic & Medicinal Chemistry Letters 4(9): 1101-1104.

Tanaka, T., Tanaka, T., Tanaka, M. (2011). Potential cancer chemopreventive activity of protocatechuic acid. J. Exp. Clin. Med. 3(1): 27-33.

Thorsen, S. (1992). The Mechanism of Plasminogen Activation and the Variability of the Fibrin Effector during Tissue type Plasminogen Activator—mediated Fibrinolysis. Ann. N. Y. Acad. Sci. 667(1): 52-63.

Tolmacheva, I., A. Tarantin, et al. (2006). Synthesis and biological activity of

nitrogen-containing derivatives of methyl dehydroabietate. Pharmaceutical Chemistry Journal 40(9): 489-493.

Unger, T. (2002). The role of the renin-angiotensin system in the development of cardiovascular disease. The American journal of cardiology 89(2): 3-9.

Vidotti, D., D. Casarini, et al. (2004). High glucose concentration stimulates intracellular renin activity and angiotensin II generation in rat mesangial cells. American Journal of Physiology-Renal Physiology 286(6): F1039-F1045.

Wada, H., S. Kodato, et al. (1985). Antiulcer activity of dehydroabietic acid derivatives. Chemical and pharmaceutical bulletin 33(4): 1472-1487.

Weisel, J. W. (2005). Fibrinogen and fibrin. Adv. Protein Chem. 70: 247-299.

Wideing, J. U., Eisinger, G., Kostering, H.J. (1989). Determination of soluble fibrin by turbidimetry of its protamine sulphate-induced paracoagulation. J. Clin. Chem. Clin. Biochem. 27(2): 57-64.

Williams, S., Fatah, K., Hjemdahl, P., Blombäck, M. (1998). Better increase in fibrin gel porosity by low dose than intermediate dose acetylsalicylic acid. Eur. Heart. J. 19(11): 1666-1672.

Yong-Hong, J. I. A., Yang, J.I.N., Qiu-Min, L.Ű., Dong-Sheng, L.I., Wan-Yu, W.A.N.G., Yu-Liang, X.I.O.N.G. (2003). Jerdonase, a novel serine protease with kinin-releasing and fibrinogenolytic activity from Trimeresurus jerdonii venom. Acta Biochem 35: 689-694.

Yoon, Y., J. Song, et al. (2000). Plasma nitric oxide concentrations and nitric oxide synthase gene polymorphisms in coronary artery disease. Clinical chemistry 46(10): 1626-1630.

Zamolodchikov, D., Strickland, S. (2012). Aβ delays fibrin clot lysis by altering fibrin structure and attenuating plasminogen binding to fibrin. Blood 119(14): 3342-3351.

Zeng, W. C., He, Q., Sun, Q., Zhong, K., Gao, H. (2012). Antibacterial activity of water-soluble extract from pine needles of Cedrus deodara. Int. J. Food Microbiol. 153(1): 78-84.

감사의 글

학부 4년과 군대 전여 후, 2007년 7월부터 시작된 대학원 생활을 시작으로 2015년 2월 졸업을 앞두고 있습니다. 실험실에서 보낸 지난 긴 시간을 돌아보면 많은 실험을 통해 고등학교 동안 꿈꿔왔던 생활을 할 수 있었습니다. 또한, 실험 외에도 많은 것을 느끼고 배우고 나 자신을 돌아보는 시간을 가질 수 있었던 그 시간이 참으로 행복했던 것 같습니다. 이렇게 무사히 학위를 마칠 수 있었던 것은 많은 분들의 도움이 있기에 가능했던 것 같습니다.

석사 및 박사과정 동안 부족했던 저를 격려와 지도 해주신 지도 교수님이신 정현숙 교수님께 진심으로 감사드립니다. 좋은 결과를 많이 가져다 드리지 못하고 졸업하게 되어 그저 죄송할 따름입니다. 좀 더 노력을 했다면 긴 대학원 생활동안 지금까지 웃을 수 있었던 것 보다 더 많이 웃을 수 있었을 것이라는 생각에 많은 안타까운 생각이 듭니다.

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양영기 교수님, 김성준 교수님, 이정섭 교수님, 박윤경 교수님, 이건호 교수님과 황인덕 박사님 그리고 실험실 후배들이 아니었다면 그 긴 시간을 버티지 못했을 것 같습니다. 먼 타국에서 화학의 기초를 잡아주신 이승서 교수님과 지금은 이곳에 있지 않지만, 항상 용기를 주신 우희권 교수님께 진심으로 감사드립니다.

대학원이라는 생활이 때론 즐겁고 흥분되기도 하지만 힘들어 쉬고 친구들과 여행도 가고 싶을때도 있었지만, 항상 친구들 사이에서 유일하게 박사학위를 받게 된 저를 격려해준 고등학교 친구 김석원, 기종, 박동현, 전호, 유동균, 정대두, 배경희, 윤상혁, 강원주 등 이곳에 다 적지 못했지만, 항상 격려해준 친구들에게 감사하다는 말을 하고 싶고, 이제는 좀 더 마음 편하게 여행가자고 말하고 싶습니다.

36년 동안 외골수에 나만 알고 살아온 저를 보살펴 주신 부모님께는 머라 감사해야 할지 모르겠습니다. 군대 전역 후 취직을 하지 않고 공부를 하겠다는 저에게 아무런 말없이 응원해주시고 지금까지 보살펴 주셔서 다시 한 번 감사드립니다.

마지막으로 저에 첫 지휘관이셨던 우승룡 대령(?)님 진심으로 감사드립니다. 부대 전입 3개월 만에 참모부로 이동하여 힘들어 하던 저에게 진심어린 충고와 보살핌으로 힘들었던 참모부 생활을 견딜 수 있게 도와주셨고 더 단단한 인격체가 되게 도와주신 대대장님께 감사드립니다.

이제 대학원 생활에서 만들어진 즐거운 추억과 가르침을 뼈 속 깊이 간직하고 이제 새로운 출발을 하려고 합니다. 부족한 부분은 채우고 좋은 점은 한 층 더 빛나게 하여 부끄럽지 않는 사람이 되도록 하겠습니다. 감사합니다.