[UCI]I804:24011-200000265192

2016년 2월
박사학위논문

Inhibitory effects of *Dendropanax morbifera* on neointima formation and myocardial injury

조선대학교 대학원

의 과 학 과

임 리 진

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황칠추출물의 신생내막형성 및 심근손상에 대한 억제 효과

2016 년 2 월 25 일

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2015년 10월

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Inhibitory effects of *Dendropanax morbifera* on neointima formation and myocardial injury

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According to the WHO, cardiovascular diseases (CVD) is the number one cause of death globally. Cardiovascular diseases include heart diseases such as, cardiomegaly, heart failure, myocardial infarction, arrhythmia and vascular diseases such as, atherosclerosis and restenosis. The development of materials derived from natural products has recently been actively conducted for the treatment of such cardiovascular diseases. The overall purpose of this study was to determine inhibitory effects of extract from *Dendropanax morbifera* (*D. morbifera)* on proliferation and migration of vascular smooth muscle cells (VSMCs) being cause of atherosclerosis and restenosis, furthermore, on cardiomyocyte apoptosis after myocardial injury. *Dendropanax morbifera Leveille* has been used in traditional medicines for various diseases such as headache, infectious diseases and general debility. However, the effect of extract from *D. morbifera* on vascular diseases and the mechanisms underlying the effects have not yet been

investigated in detail. First of all, to determine the working concentration, MTT assay and BrdU assay were performed. The proliferative activities were significantly decreased by ~40% in RAoSMCs treated 25 μg/ml of *D. morbifera*, which has been determined as the working concentration in further study. In 2-D and 3-D migration assay, the treatment of extracts from *D. morbifera* significantly decreased the RAoSMCs migration by more than 40% and ~50% compared to control. Treatment of the extracts from *D. morbifera* significantly reduced the mRNA levels of matrix metalloproteinase (MMP) 2 and 9 whereas the levels of MMP 7 were not altered. Also western blot assay results through both the media and cell lysate showed that *D. morbifera* significantly reduced the platelet derived growth factor (PDGF)-induced MMP2 and 9 expression. Similar results were observed with gelatin zymographic assay. Also the phosphorylated levels of AKT and ERK 1/2 were increased in PDGF-treated RAoSMCs, which has been selectively decreased in *D. morbifera*-treated RAoSMCs. These results demonstrate that Akt and ERK are likely a downstream target of *D. morbifera*-mediated signaling that regulates MMP2 and MMP9 expression and their functions in cell migration in RAoSMCs. Further, neointima formation in balloon-injured rat carotid artery were significantly decreased in the extracts from *D. morbifera*-treated rats by 50~60% compared to non-treated controls. Also immunohistochemical analysis for balloon-injured vessels of the extracts from *D. morbifera*-treated rats using ki-67 and PCNA antibodies revealed a significant reduction of the proliferative activity in the neointimal layer. Western blot analyses for tissue lysates showed similar results of 50~70% decrease compared to controls. In addition, immunohistochemistry of balloon-injured vessels in the extracts from *D. morbifera*-treated to rats with antibody to MMP2, 9 revealed with a reduction of the migratory activity in the neointimal layer. Furthermore, the expression level of MMP2 were significantly reduced in the extracts from *D. morbifera*-treated to rats by 60~75% compared to controls. These results suggest that the

extracts from *D. morbifera* inhibit proliferation and migration in RAoSMCs also reduce neointima formation after balloon injury in rats. In this study, the effects of the extracts of *D. morbifera* on hypoxia/reoxygenation (H/R) injured cardiomyocytes (CMC) were investigated. The condition of cardiomyocytes with H (30min)/R (1hr) decreased the cell number, but the treatment of *D. morbifera* inhibited CMC death induced apoptosis. These results suggested that the progress of apoptosis in CMC are regulated by *D. morbifera* after H/R. *D. morbifera* treated CMC reduced ROS generation and intracellular calcium concentration (Ca^{2+}_i) compared with positive control (the condition of H/R). Also DM attenuated abnormal changes of RyR2 and SERCA2a genes in hypoxic cardiomyocytes by western blot. These results suggest that DM ameliorates ROS generation and Ca^{2+}_i homeostasis as preventing dysregulation of calcium regulatory proteins in the heart, thereby producing the cardioprotective effect and reduction in hypoxic cardiomyocytes damage. In order to screen the natural products having activities for cardiovascular diseases, 31 more extracts besides *D. morbifera* were tested. Then, each extracts were tested whether they have anti-proliferative and anti-migratory activities on RAoSMCs. As these results, indicated that the activity of *Plantago asiatica* is specific on anti-proliferation and those of *Pinus densiflora* is specific on anti-migration in RAoSMCs. Further studies will be needed to reveal the mechanisms underlying the effects of the extracts on the regulation of RAoSMCs. Because MMP13 is associated with tumor cells migration, hypothesized that MMP 13 participates in VSMC migration induced by certain stimuli such as PDGF and angiotensin II (Ang II). This study found that the mRNA level of MMP13 in RAoSMCs was increased by both PDGF and Ang II. Also observed the significant decrease of migration in PDGF- or Ang II-treated RAoSMCs by MMP13 specific inhibitor treatment. Silencing of MMP13 by a specific small interfering RNA (siRNA) significantly decreased expression of the active form of MMP13, which is followed by the decreased migration of PDGF-or Ang II-treated

RAoSMCs. Interestingly, the study observed synergistic inhibitory effects on migration by treatment with MMP2 and 13 or MMP9 and 13 inhibitors compared with that in single treatments. Moreover, found that cordycepin, a known inhibitor of VSMC migration, caused significant downregulation of MMP2, 9, and 13 expression in PDGF-treated RAoSMCs. To understand the mechanism by which stimuli regulate MMP13 expression in VSMCs, analyzed the effects of signal mediator inhibitors on MMP13 expression and found that an Akt or ERK-specific inhibitors, but not PI3K inhibitor, significantly decreased MMP13 expression levels. Together, these study strongly suggest that MMP13 involves VSMCs migration via an Akt and ERK-dependent regulation.

국문초록

황칠추출물의 신생내막 형성 및 심근 손상에 대한 억제 효과

최근 WHO 통계에 따르면, 심혈관계 질환은 전 세계적으로 사망원인 1위인 질환이다. 심혈관계 질환은 심비대증, 심부전, 심근경색, 부정맥 등과 같은 심장질환과 동맥경화나 재협착과 같은 혈관질환을 포함한다. 이러한 심혈관계 질환을 치료하기 위한 천연물 유래의 소재 개발이 최근 활발히 진행되고 있다. 본 연구에서는 황칠나무의 추출물이 동맥경화와 재협착의 원인이 되는 혈관평활근세포의 이동과 증식을 억제할 수 있는 지와 심근경색의 원인이 되는 심근세포 사멸을 억제할 수 있는 지를 확인하였다. 황칠나무는 두통, 염증성 질환, 신경쇠약 등과 같은 다양한 질환을 위한 전통 의약으로 사용되어 왔으나 심혈관질환에 대한 황칠추출물의 효과 및 그 기전은 밝혀진 바가 없다. 본 연구에서는 먼저 MTT assay와 BrdU assay를 시행하여, 25μg/ml의 황칠추출물이 혈관평활근세포의 유의적인 증식억제 효과를 보여주는 것으로 확인한 바, 이 후 이 농도를 실험에 사용하였다. 황칠추출물의 처리시 2D와 3D 이동성 분석에서 공통적으로 혈관평활근세포의 유의적인 이동억제효과를 확인하였으며, 황칠추출물을 처리한 실험군에서 세포이동의 지표인 MMP2, 9의 발현변화를 q-PCR, western blot, zymographic analysis를 통해 확인한 바, MMP2와 9 모두 황칠 처리군에서 유의적인 감소를 보여줌으로서 이는 황칠추출물의 혈관평활근세포 이동 억제효과가 MMP2, 9에 특이적이라는 것을 확인할

수 있었다. 또한 세포의 증식과 이동에 중요한 신호전달 물질로 알려진 AKT와 ERK의 인산화정도를 western blot을 통해 분석한 결과, 인산화된 AKT와 ERK의 양이 황칠추출물을 처리한 실험군에서 대조군과 비교시 현저하게 감소한 것으로 나타났으며, 이러한 연구결과들을 통해서, 황칠추출물이 MMP2와 9에 특이적으로 AKT와 ERK의 신호전달체계를 통해 PDGF에 의해 유도된 혈관평활근세포의 이동과 증식을 억제하는 효과를 가진다고 제안한다. 더 나아가 동물모델의 H&E staining 결과에서, 4주간 황칠추출물을 전혀 먹이지 않은 대조군은 풍선확장술 시행 후 현저한 혈관내막형성이 진행되었으나 풍선확장술 전 2주간 황칠추출물을 먹인 실험군은 대조군과 비교시 약 60%의 혈관내막형성 억제율을 나타내었으며 N/M ratio 역시 대조군과 비교시 54%로 현저히 감소됨을 확인하였다. 면역조직화학법을 통해 황칠추출물의 혈관평활근세포 증식과 이동억제효과를 확인한 바, 세포증식의 지표인 PCNA와 Ki-67의 증식활성의 감소를 확인하였으며, 수축성 혈관평활근세포의 지표인 α-SMA의 유의적인 증가를 확인하였고, 세포이동의 중요한 지표인 MMP2, 9 활성의 유의적 감소를 확인하였다. 조직 표본에서 시행한 western blot과 q-PCR 결과에서도 MMP2와 PCNA의 발현이 현저하게 감소한 것으로 확인되어, 황칠추출물의 증식과 이동억제효과를 다시 확인할 수 있었다. 심근세포의 허혈손상에 대한 황칠추출물의 효과를 확인하기 위하여, 먼저 저산소 30분/재산소 1시간씩 처리된 심근세포에 결정된 농도의 황칠추출물을 처리하였는데, 대조군에 비해 유의적인 사멸억제효과를 보여주었다. 심근세포의 허혈/재관류 손상에 따른 ROS양을 측정한 결과, 황칠추출물을 처리했을 때 발생하는 ROS의 양이 현저히 감소하여 약 50%이상의 ROS 감소효과를 보여주었으며, 허혈/재관류 손상으로 세

포질내에 증가된 칼슘이온농도가 황칠추출물을 처리했을 때 세포질내 칼슘농도가 감소하는 것을 확인할 수 있었다. 또한 칼슘항상성을 유지하는 가장 중요한 채널인 SERCA2a와 RYR2의 경우, 허혈/재관류손상으로 인해 감소되었던 SERCA2a와 증가되었던 RYR2가 황칠추출물이 처리된 세포에서 정상적인 수준으로 회복되는 것을 확인하였다. 이러한 결과들을 통해 황칠추출물이 심근세포에서 허혈/재관류 손상에 의한 칼슘채널의 변화를 통해 칼슘항상성을 유지하고, ROS 발생억제효과를 통해 심장보호 효과를 가진다고 제안한다. 황칠추출물에 추가로 심혈관계 질환에 효과가 있는 천연소재를 탐색한 결과, 질경이의 뿌리가 혈관평활근세포의 증식억제효과를 보이며, 소나무 껍질이 혈관평활근세포의 이동억제효과를 보이는 것으로 확인되어 각각의 좀 더 자세한 작용기전을 밝히기 위한 추가연구를 진행 중이다. 또한, 최근 유방암이나 피부편평상피세포암의 이동과 전이에 MMP13이 중요한 매개인자로 밝혀지면서 혈관평활근세포의 이동에서 MMP13의 역할을 밝히기 위한 실험을 진행하였다. MMP 특이적 억제제를 처리하였을 때, 2D와 3D 이동 실험 모두에서 MMP2와 9과 마찬가지로 MMP13도 이동억제효과를 보여주었으며, siRNA로 MMP13 발현을 억제한 세포의 이동이 억제됨을 확인하였다. 이동억제제로서 기존 논문에서 입증된 cordycepin을 처리하여 세포이동을 억제한 후 MMP13의 발현을 확인한 western blot과 q-PCR 결과에서, MMP2, 9뿐만이 아니라 MMP13도 감소되는 결과를 얻을 수 있었다. 추가적으로 AKT와 ERK가 MMP13의 신호전달체계임 확인하여, 이 같은 결과들을 통해, MMP2, 9에 이어 MMP13도 AKT와 ERK의 인산화를 통해 혈관평활근세포의 이동에 관여함을 확인하였다.

1. Introduction

Cardiovascular disease (CVD) is a class of diseases that involve the heart or blood vessels. CVD is the number one cause of death globally [1]. According to the WHO, an estimated 17.5 million people died from CVD in 2012, it is expected to increase to 23.6 million people in 2030 [2]. The proliferation and migration of abnormal vascular smooth muscle cells (VSMCs) are critical events in the development of CVD such as atherosclerosis and restenosis after vascular injury [3, 4]. Following vascular injury occurrence, VSMCs proliferate and migrate in the intima in response to various factors and secrete several proteases and extracellular matrix proteins that form neointimal hyperplasia and atheromatous plaques under the influence of cytokines and growth factors, such as vascular cell adhesion molecules, pro-inflammatory mediators, and matrix molecules [4, 5]. Various molecules, involving platelet-derived growth factor (PDGF), angiotensin II (Ang II), serum and thrombin, are well known to promote the migration and proliferation of VSMCs and to form pathological neointimal hyperplasia in vascular lesion [6, 7]. In these molecules, PDGF induces activation of mitogen-activated protein kinases (MAPKs) and downstream signaling proteins, such as ERK or PI3K/Akt [8]. Furthermore, the degradation of the extracellular matrix and basement membrane by proteases, such as matrix metalloproteinases (MMPs) is significant for atherogenesis initiated by VSMCs [9, 10]. Particularly, MMP2 and MMP9 are highly expressed in migratory and proliferative VSMCs [11-14]. Also high occurrence of restenosis after percutaneous transluminal coronary angioplasty (PTCA) remains a problem to long-term successive effects of the therapy [15]. Although drug-eluting stents (DES) can effectively reduce restenosis, they does not resolve an increased the late stent thrombotic risk [16, 17]. As noted above, artery injury following PTCA

derive growth factors such as PDGF and these induce VSMC proliferation and migration by phosphorylation of downstream signaling protein such as MAPK pathway [18]. Furthermore in VSMCs, over production of reactive oxygen species (ROS) promote the proliferation and migration of medial VSMCs, leading to neointima formation and restenosis [19-21]. Thus inhibiting the proliferation and migration of VSMCs may be therapeutic mediation to attenuate the restenosis.

It is within bounds to say that numerous hormone or various ingredients to play an important roles in our bodies exist in the plant of the world [22, 23]. Recently the use of synthetic medicines for the blocking of atherosclerosis is not enough because of the limited indications for bad side effects and high cost in the treatment [24]. Definitely, effectual anti-atherosclerotic medicines based on natural products constitute a preferred alternative [25]. Many of epidemiological research have showed that diets rich in vegetables and fruits are continuous with a diminished risk of cardiovascular diseases [26-29]. In particular, their high content of polyphenols may preserve the cardiovascular system by preventing platelet aggregation and SMC migration and proliferation [30]. Among the many natural products, widely known throughout the east and west, polyphenol (catechin, quercetin, resveratrol) contained in the green tea, cordycepin, tumeric (curcumin), ginger (gingerol), tomato (lycopene) and so on were reported that are effective natural medicines against cardiovascular disorder. According to reports, the green tea catechins including Epigallocatechin-3-gallate (EGCG) inhibited intimal hyperplasia through ERK suppression in the wire-injured carotid artery and demonstrated that the EGCG protected on ischemia/reperfusion injury through STAT-1 activity in isolated rat hearts [31, 32]. Resveratrol which mainly exists in red grapes and wine, protected cardiomyocytes against ischemia/reperfusion injury through the TLR4/NF-Kb signaling pathway [33]. Lycopene, a carotenoid compound, found naturally in tomato protected

against hypoxia/reoxygenation by preventing mitochondrial dysfunction in cardiomyocytes [34]. Also, allicin, garlic component attenuated the intracellular reactive oxygen species (ROS) increase induced by H_2O_2 on cardiomyocytes [35]. Curcumin, a major active component of turmeric, investigated inhibition of platelet aggregation in a tail thrombosis model and protective effect on myocardial ischemia reperfusion injury by improving myocardial metabolism [36, 37]. Furthermore, cordycepin, a main compound derived from *cordyceps militaris,* inhibited vascular smooth muscle proliferation through Ras/ERK1 pathways and decreased the infarct size in left ventricle [38-40]. The plant resources having activities for cardiovascular diseases were listed in Table 1.

In Korea, the plant *Dendropana morbifera leveille* (*D. morbifera*, family araliaceae) is commonly used as a traditional medicine for various diseases and healthy food including general debility. Also it is well known to be a producer of golden varnishes, which is distributed in the sourthern part of Korea [41]. *D. morbifera* is also known to be effective for treatment of several dieases such as infectious diseases, skin diseases, diabetic, atherogenic, plasmoidal, cancer [42-44]. However, the effects of extracts from *D. morbifera* on the cardiovascular diseases have not yet been reported. In the present study, showed the antiproliferative and antimigratory effects of the extracts from *D. morbifera* on VSMCs *in vitro* [45], inhibitory effects of neointima formation *in vivo*, and attenuating effects of myocardial injury caused by increased ROS and calcium ion cincentration.

Plus, did quite a lot of research made in order to find out natural products related to cardiovascular disease. In a recent research, phytoncide, the stem oil extracted from *Chamaecyparis obtuse* (C. *obtuse*) is proved to have many biological activities including antimicrobial activity, antioxidant and NK cell activating activity [46, 47]. In previous study, phytoncide, nanochemicals from C. *obtuse* (received from Forest resources institute, South

Table 1. Natural sources reported as anti-cardiovascular diseases.

Cardiovascular diseases	Sources	Main compounds	Outcome/mechanisms
Platelet aggregation	*Ziziphus jujube* [48]	Jujuboside B	Blocks TXB_2 formation
	Gardenia jasminoides [49]	Geniposide, Genipin	Inhibits PLA_2
	Oenanthe javanica [50]	IMG, hyperoside Flavone	Inhibits production of thrombin, FXa, and TNF-α
	Hippophae Rhamnoides L [51]		Inhibits platelet aggregation
	Cornus officinalis [52]	Cornuside, bisiridoid glucoside	VSMCs dilation via NO/cGMP signaling
	Paeonia lactiflora [53]	Paeonol, paeoniflorin, Benzoylpaeoniflorin, etc	Platelet anti-aggregatory and blood anticoagulant effects
Hypertrophy	*Allium sativum* [54]	Allicin, allin	Antihypertrophic action by increased cellular NO and H_2S
Hypertension	*Undaria pinnatifida* [55]		Hypotensive effect
	Eucommia ulmoides Oliv. [56]	lignans	Reverse hypertensive vascular remodeling
	Saururus chinensis [57]		Reduced left ventricle pressure
	Gardenia jasminoides [58, 59]	Crocetin, Carotenoid	Attenuates EC dysfunction via NO decrease
	Hippophae Rhamnoides L. [60]	Que, Isor	Dilatation of vascular and hypotensive effect
	Carthamus tinctorius [61]	SY(safflower yellow)	Plasma renin activity and angiotensin II level diminished
	Cynanchum wilfordii [62]		Ameliorates hypertension via improvement of NO/cGMP signaling

Table 1. Continue.

Atherosclerosis	Green tea [31]	EGCG	Inhibits intimal hyperplasia via ERK suppression
	Cordyceps militarys[38, 39]	Cordycepin	Antiproliferation via Ras/ERK1 pathways
	Nelumbo nucifera [13]		Anti-proliferative, -migratory action via inhibition of ERK1/2 and MMP2,9
	Magnolia officinalis[63-65]		Attenuates intimal hyperplasia
	Rubus coreanus [66]		Attenuates atherosclerosis by improving blood lipid profile
	Panax ginseng [67]		VSMC antiproliferation via suppressing Jak/Stat pathway
	Dendropanax morbifera [45]		Attenuates VSMC migration via inhibition of MMP2,9
	Diopyros kaki[68]		Attenuates HASMCs migration and invasion via inhibition of c-Src activity
Myocardial ischemia/reperfusion injury	*Cordyceps militaris*[40]	Cordycepin	Decreased the infarct size in left ventricle
	Tomato[34]	Lycopene	Preventing mitochondrial dysfunction via inhibiting the increase of MDA levels,
	Graph[33, 69]	Resveratrol	Protects cardiomyocytes
	Cornus officinalis[70]	Cornuside	Decrease of infarct volume
	Carthamus tinctorius[71, 72]	SY(safflower yellow)	Inhibits oxidative stress
	Allium sativum[35]	Allicin	ROS scavenger
	Green tea[32, 73]	EGCG	Inhibition STAT-1 activity
	Curcuma longa L.[37]	Curcumin	Augmentation of endogenous antioxidants and improving myocardial metabolism
Arrhythmia	*Oenanthe javanica*[74]		Decrease ventricular fibrillation

Jeolla Province) that was identified with inhibitory effects through anti-proliferation and anti-migration of VSMCs [75]. Besides *D. morbifera*, 31 more extracts, which were selected through a search of the relevant literature, have been tested whether they have activities for CVD in this study. Then, each extracts were tested whether they have anti-proliferative and anti-migratory activities on RAoSMCs.

As it seems to be active for CVD, *Pinus densiflora* has been used as a folk therapy for hemorrhage, rheumatism, hypertension, gastroenteritis and asthma [76]. Recently, the studies have shown that PS have an antioxidative, anti-inflammatory, antitumor effects [77-80]. However, the effects of PS on the cardiovascular diseases have not been identified. Also *Plantago asiatica* is a traditional medicine in East Asia, used to treat infectious diseases, digestive disorders and tumours and recent studies have offered some potential mechanisms of the hermetic effect of *P. asiatica* extracts [81]. It was suggested that treatment with *P. asiatica* water extracts induced haeme oxygenase-1 (an inducible stress protein-degrading enzyme) expression in cultured cells without cytotoxicity [82]. In addition, hot water extracts of *P. asiatica* showed significant inhibitory activity toward the proliferation of lymphoma and carcinoma cells and to herpes and adenoviral infection. *P. asiatica* water extracts also enhance interferon-γ secretion in human mononuclear cells [83]. As these results, indicated that the activity of *Plantago asiatica* is specific on anti-proliferation and those of *Pinus densiflora* is specific on anti-migration in RAoSMCs using BrdU assay in this study and gelatin zymographic aanlysis (data not shown). Further studies will be needed to reveal the mechanisms underlying the effects of the extracts on the regulation of RAoSMCs.

Cordycepin is a type of nucleoside analogue isolated from *cordyceps militaris*, which is reported to inhibit VSMCs migration through downregulation of MMP2 and MMP9 [38].

Because MMP13 is associated with tumor cells migration, we hypothesized that VSMCs migration induced by certain stimuli such as PDGF and Ang II, is mediated through upregulation of MMP 13. We found that cordycepin, known to inhibitory chemicals on VSMCs migration, significantly decreased RAoSMCs migration via downregulating MMP 13 expression in PDGF-induced RAoSMCs [84].

Taken together, these finding suggest that *D. morbifera* is an effective therapeutic agent for treating neointima formation and myocardial injury. Furthermore, these finding suggest that the extract of natural products including *Pinus densiflora and Plantago asiatica* are effective supplements to prevent cardiovascular disease.

2. Materials and Methods

2.1. Preparation of the extracts of natural plants including *D. morbifera*

For preparation of extracts, briefly, dried leaves 1.4g and stems 21g from *D. morbifera* was extracted in 500ml of distilled water at 60℃, for 24hr. After high-speed centrifugation and filtration under sterile conditions, obtained the filtrate were sterilized at 125℃ for 30min. The resultant extracts of *D. morbifera* were stored at 4℃ and diluted by 50X in distilled water immediately prior to the experiments. In addition, several decades of natural plants have been tested in order to screen the additional natural products having the activities for cardiovascular diseases. They are listed in Table 2. Samples designated by No. 1~23 were obtained from Korea plant extract bank and *Pinus densiflora* (No. 24~31) were extracted using the same method using the designated solvents described above for *D. morbifera*.

2.2. Primary culture of rat aortic smooth muscle cells

Rat Aortic Smooth Muscle Cells (RAoSMCs) were isolated from 6- to 8-week-old Sprague-Dawley rats. The thoracic aortas were removed and immediately placed in a 100mm cell culture dish containing serum-free DMEM (containing 0.1% penicillin, WelGENE). The aorta was freed from connective tissue and any remaining clotted blood was removed. The aorta was then severed and transferred into a tube containing a mixture of collagenase type I and elastase (1 and 0.5mg/ml, respectively), and incubated for 30min at 37℃. Piece of aorta

Table 2. Additional natural products used in this study

No.[a]	Scientific name (Korean)	Parts[b]	Extraction solvent
1	*Chrysanthemum zawadskii* (구절초)		Me-OH 99.9%
2	*Torilis japonica* (사상자)		Me-OH 99.9%
3	*Pleuropterus multiflorus* (하수오)		Me-OH 99.9%
4	*Cardamine leucantha* (미나리냉이)		Me-OH 99.9%
5	*Allium tuberosum* (부추)		Me-OH 99.9%
6	*Platycodon grandiflorum* (도라지)		Me-OH 99.9%
7	*Eucommia ulmoides* (두충)	Stem	Me-OH 99.9%
8		Leaves	Me-OH 99.9%
9		Bark	Me-OH 99.9%
10		Seed	Me-OH 99.9%
11	*Diospyros kaki* (감나무)	Stem	Me-OH 99.9%
12		Bark	Me-OH 99.9%
13		Root	Me-OH 99.9%
14	*Plantago asiatica* (질경이)		Me-OH 99.9%
15		Aboveground	Me-OH 99.9%
16		Root	Me-OH 99.9%
17	*Gardenia jasminoides* (치자)	Leaves	Me-OH 99.9%
18		Stem	Me-OH 99.9%

Table 2. Continue

19	*Cornus officinalis* (산수유)	Leaves, Stem	Me-OH 99.9%
20		Seed	Me-OH 99.9%
21	*Cuscutae Semen* (토사자)		Me-OH 99.9%
22	*Pinus densiflora* (소나무)	Leaves	Me-OH 99.9%
23		Bark	Me-OH 99.9%
24	*Pinus densiflora* (소나무)	Bark	Me-OH 99.9%
25		Bark	Et-OH 99.9%
26		Bark	Ionic water
27		Bark	Distilled water
28		Leaves	Me-OH 99.9%
29		Leaves	Et-OH 99.9%
30		Leaves	Ionic water
31		Leaves	Distilled water

[a]Samples designated by No. 1~23 were obtained from Korea plant extract bank and 24~31 were extracted using the method described in Materials and Methods section.

[b]Blank indicates total body of the plant.

was placed into a 100mm cell culture dish and stripped off adventitia with forceps under a binocular microscope. And then each piece of aorta was severed once more and transferred into a tube containing 5ml of the enzyme dissociation mixture (containing collagenase and elastase). And the tube were incubated for 2h at 37℃. Dispersion of the tissue was accomplished by pipetting slowly. The suspension was centrifuged (1600 rpm for 5min) and the pellet was resuspended in DMEM with 10% FBS. And was recentirifuged (1600 rpm for 5min). Cells were cultured over several passages (up to 10) in a humidified atmosphere of 95% air and 5% CO_2 incubator at 37℃. PDGF was obtained from Sigma.

2.3. Primary culture of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated and purified by the modifications of Horikawa, et al's method. Briefly, 2-3 day old Sprague-Dawley rat pups were disinfected with povidone and then dissected. The chest was opened and the heart was rapidly removed and washed with the Phosphate-buffered saline solution (pH 7.2, WelGENE) lacking Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors, hearts were minced until the pieces were approximately 1mm3 and treated with 5ml of collagenase type II (0.9mg/ml, 210units/mg, Gibco BRL) for 7min at room temperature. The cells in the supernatant were transferred to a tube containing cell culture medium (α-MEM containing 10% fetal bovine serum, WelGENE). The tubes were centrifuged at 1200 rpm for 4min at room temperature, and the cell pellet was resuspended in 3ml of cell culture medium. The above procedures were repeated 6-8 times until there little tissue was left. Cell suspensions were washed twice with cell culture medium and seeded to achieve a final concentration of 5×10^5 cells/ml and then they were plated onto gelatin-coated 6well plates.

The cells were cultured in α-MEM containing 10% fetal bovine serum with 0.1mM Bromodeoxyuridine (Brdu) which was used to prevent proliferation of cardiac fibroblast. Cells were then cultured in 5% CO2 incubator at 37℃.

2.4. Cell viability assay

RAoSMCs were plated in triplicate wells of 96-well plates at a density of 4×10^3 per well and treated with the designated concentrations of the extracts. The extracts were dissolved in distilled H_2O. Cell viability was assessed with the MTT assay. After the incubation period, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma, St. Louis, MO, USA) was added to each well to a final concentration of 0.5 mg/ml and was incubated at 37 ℃ for 3 h to allow MTT reduction. The formazan crystals were dissolved by adding dimethylsulfoxide (DMSO), and absorbance was measured at 570 nm with a spectrophotometer. Experiments were performed in triplicate.

2.5. Cell proliferation assay

Cells on 96-well plates were treated with the designated concentrations of the extracts, then the BrdU proliferation assay was performed after 24 h using the cell proliferation colorimetric ELISA system (Promega) according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plate $3x10^3$ cells/well. After 24 h of incubation, the designated concentration of extracts of natural plants were treated for 24 h. Then, cells were treated with fixing/denaturing solution at room temperature for 30 min. Consequently, cells were treated

with detection antibody solution, HRP-conjugated secondary antibody solution, and TMB substrate. Then, the absorbance at 450 nm were recorded. Experiments were performed in triplicate.

2.6. Cell migration assay

Cell migration activities were examined by three-dimensional Boyden chamber assay and two-dimensional wound healing assay. For Boyden chamber assays, cells were placed in the upper compartment of the Transwell chambers coated with collagen I on the lower surface (5×10^4 cells in 100 μl). The extracts from *D. morbifera* were treated with the designated concentration in each upper compartment. After incubation for 16 h at 37°C, cells on the lower surface of the filter were fixed and stained, and five random fields/membrane were counted at x200 magnifications. For wound healing assay, a rectangular lesion was created using a cell scraper, and then the cells were incubated with designated concentration of the extracts from *D. morbifera* for designated times. The distance from the margin of the lesion to the 10 most migrated cells were measured, and the mean value of the distances was taken as the mobility of cells in each culture dish.

2.7. Gelatin zymography

Gelatinase activity in the conditioned medium collected from cell cultures was measured by zymography. This procedure has been shown to quantitatively estimate both proenzyme and activated MMP enzyme activity. Briefly, aliquots of the control and test media were

electrophoresed on a 10% SDS-polyacrylamide gel containing 0.8% gelatin. Gels were washed with 2.5% triton X-100 to remove SDS (30min, two times), washed with D.W for 1hr and then incubated at 37°C for 48hr in developing buffer (50mm Tris-HCL, pH 7.5, 5mM $CaCl_2$, 1μM $ZnCl_2$, 0.02% sodium azide, 1% Triton X-100). After 48hr, the zymographic activities were revealed by staining with 1% Coomassie blue and later, destaining of the gel and were quantified by laser densitometry of the corresponding bands in the linear response of the gelatin zymogram.

2.8. Immunoblot analysis

Cells were washed in PBS and lysed in RIPA buffer (containing 1mM PMSF, protease inhibitor cocktail). Protein concentrations were determined using the Bradford protein Assay kit (Bio-Rad, Hercules, CA). Equal quantities of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad laboratories. Inc.). After membrane blocking with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% or 10% skim milk for 1 hour at room temperature, the membrane was incubated with primary antibody for overnight at 4°C. The primary antibodies were used at the following dilutions in blocking buffer: MMP2 (1:2000, Abcam), MMP9 (1:500, Abcam), phospho Akt (1:200, Cell Signalling Technologies), Akt (1:1,000, Cell Signalling Technologies), phospho ERK (1:1,000, Cell Signalling Technologies) , ERK (1:1,000, Santa Cruz) , β-actin (1:5,000, Sigma), PCNA (Cell Signaling, 1:2000), Ki-67 (Thermo, 1:2000). The membrane was washed five times with TBS-T for 5 minutes and incubated for 1hour 30minunte at room temperature with secondary

antibodies. After extensive washing, bands were detected by enhanced chemiluminescence reagent (ECL, BIONOTE, Animal Genetics Inc.). Band intensities were quantified using the Image J quantification software.

2.9. Real-time quantitative PCR (qPCR)

Total RNA was isolated using Trizol reagent (QIAGEN, Valencia, USA) according to the manufacturer's protocol. The RNA concentration of each sample was measured by a spectrophotometer (TECAN) at 260nm. Total RNA was subjected to reverse transcription using HelixCript™ 1st-Strand cDNA Synthesis Kit (NanoHelix). Real-time quantitative PCR with realHelix™ qPCR kit (NanoHelix) was performed by the SYBR Green method using an Applied Rotor-Gene 3000^{TM}. The ratios of the transcript levels of genes of interest in experimental and control sample were compared with the ratios of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) transcript levels in corresponding samples. Table 3 was primer sequences for qPCR.

2.10. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS were measured using fluorescent dye technique. Cells were seeded into a 24well plate with glass cover slips at a density of 5×10^4 cells/ml and cultured for 24h. Then, cells were treated with negative control (DMSO), positive control (H_2O_2 200nM), and the extract of *D. morbifera* with dose dependent manner for 1h. Then, cells were washed twice with calcium free PBS (PBSc) and loaded with 2',7'-dichlorofluorescin diacetate (H_2DCFDA,

Table 3. The sequence of primers used for real-time quantitative PCR

Gene	Primer sequence
GAPDH	Sense: 5'-CAGTGCCAGCCTCGTCTCAT-3' Antisense: 5'-TGGTAACCAGGCGTCCGATA-3'
MMP2	Sense: 5'-ACGATGGCAAGGTGTGGTGT-3' Antisense: 5'-CCTTGGTCAGGACAGAAGCC-3'
MMP7	Sense: 5'-AACTGGTATTGTGCTGGACTCTGG-3' Antisense: 5'-CACGGACGATCTCACGCTCAG-3'
MMP9	Sense: 5'-CGAGCTATCCACTCATCAAACAT-3' Antisense: 5'-GTGTCCTCCGATGTAAGAGAGAA-3'
MMP13	Sense: 5'-GCGGGAATCCTGAAGAAGTCTAC -3' Antisense: 5'-TTGGTCCAGGAGGAAAAGCG -3'
PCNA	Sense: 5'-TGAAGTTTTCTGCGAGTGGG -3' Antisense: 5'-CAGTGGAGTGGCTTTTGTGAA -3'
siMMP13(#1)	Sense: 5'-GACAUCAUGAGAAAACCAAtt -3' Antisense: 5'-UUGGUUUUCUCAUGAUGUCtt -3'
siMMP13(#2)	Sense: 5'-CUGUGAAGCUUCAGUAtt -3' Antisense: 5'-UACUGAAGCUUGUUCACAGtt -3'
siMMP13(#3)	Sense: 5'-CACACUGAUAGAGGACACAtt -3' Antisense: 5'-UGUGUCCUCUAUCAGUGUGtt -3'

Invitrogen, USA) and 4',6-diamidino-2-phenylindole (DAPI) diluted with calcium free warm PBS to a final concentration of 10μM and 50μg/ml, respectively. After, cell were incubated for 10min at 37℃ in dark. The probe H_2DCFDA (10μM) entered into the cells, and the acetate groups on the H_2DCFDA were cleaved by cellular esterases, trapping the nonfluorescent 2',7'-dichlorofluorescin (DCFH) within the cells. Subsequent oxidation by reactive oxygen species yielded the fluorescent product DCF. Then, cells were gently washed the coverslips three times in warm PBS and the coverslips were placed in the chamber, which was mounted on the stage of an inverted microscope equipped with a confocal laser-scanning system. The dye, when exposed to an excitation wavelength of 480nm, emitted light at 535nm only when it had been oxidized. Fluorescence images were collected using a confocal microscope (Fluoview FV1000 confocal system, Olympus) by excitation at 488nm and emission greater than 500nm with a long-pass barrier filter. The fluorescence intensity of an equivalent field size (3X3 mm) in plate was measured using the Image J quantification software.

2.11. Measurement of intracellular Ca^{2+} alteration

The intracellular calcium was measured using the fluorescent calcium indicator, Fluo-4am (Invitrogen, USA). Cardiomyocytes were seeded into a 4well chamber at a density of 1×105 cells/ml and cultured for 24h. Then, Cell were treated with negative control (DMSO), SNU-115, SNU-62 with dose dependent manner for 20min. Cell were washed with serum free medium (α-MEM, WelGENE) and loaded with Fluo-4am diluted with serum free medium to a final concentration of 2μM and incubated for 20min at 37℃ in dark. Then, cells were washed twice with warm PBS and covered to the cover side. Fluo-4am fluorescence imaging was performed

using confocal microscopy (Fluoview FV1000 confocal system, Olympus). Fluo-4am was excited with the laser at 488nm, and fluorescence was measured at a wavelength of 515nm.

2.12. Balloon injury animal model

Rat (white Sprague-Dawley, 6-7 weeks old, body weight 200±50 g) were anesthetized by intramuscular injection of 20 mg kg−1 Zoletil 50® (Virbac Corp., Fort Worth, TX, USA) with 10 mg kg−1 Rompun® (Bayer Corp., Pittsburgh, PA, USA). After midline incision of the the neck, the left external carotid artery was exposed and was injured using 2F balloon catheter (Edwards Lifesciences) introduced up to the aortic outlet of the common carotid artery with inflation and deflation to expand the artery and denude endothelium. This procedure was repeated three or four times. Afterwards, the catheter was removed, the external carotid artery ligated and the wound was closed. The contralateral common carotid arteries served as uninjured controls. 14 days after surgery rat were anaesthetized as described previously, Blood was collected from the inferior vena cava, stored at -80℃ deep-freezer for Multiplex analysis of serum. For morphometric analysis of neointima formation by Hematoxylin and Eosin staining (H&E staining) and Immunohistochemistry (IHC), bilateral carotid arteries were excised, adhering connective tissue and remaining blood was removed and stored at 4% paraformaldehyde (PEA) for fixation. Or excised bilateral carotid arteries were used for western blot and q-PCR. The extract of *D.morbifera* was orally administered (90mg/kg/d), starting two weeks before surgery and continuing for two weeks after carotid injury.

2.13. Morphometric analysis

The vessels of rat were harvested on 14 days after balloon injury as previously described. The injured section of common carotid artery were fixed in 0.4% formalin and were embedded in paraffin, and 6-μm cross-sections were cut. And then sections from each vessel were stained with hematoxylin and eosin (H&E). The cross-sectional areas of the blood vessel layers including the lumen area (LA), intimal area (IA), and medial area (MA) were quantified at least 3 different sections (proximal, middle, and distal) by using Image J. The Intima to media ratio (I/M ratio) was calculated from the mean of these determinations. Data are presented as means ±S.D. from four animals in each group.

2.14. Immunohistochemistry

For assessment of proliferation in the vessel wall, cross-sections were stained by immunohistochemistry using an antibody directed against proliferating cell nuclear antigen (PCNA), Ki-67, MMP2 and MMP9. Paraffin-embedded sections were hydrated using xylene and successive ethanol (100%, 95%, 80%, 70%) baths. Sections were incubated with antigen unmasking solution (citrate or Tris-EDTA buffer, at 100℃, 20 min), and washed in water, H_2O_2 (3%, 5 min) and the treated at room temperature with standard blocking serum (10% normal horse serum in PBS) supplemented with 0.1% Triton X-100 for 20 min. Sections were then incubated with respective monoclonal primary antibodies (overnight, 4℃) as follows: antibodies against PCNA (Cell Signaling, 1:1000), Ki-67 (Thermo, 1:100), MMP2 (Abcam, 1:500), MMP9 (Abcam, 1:100). Sections were then washed in PBS and incubated with

appropriate diluted biotinylated secondary antibody solution (1/200) at room tempeature for 1 hour and washed in PBS. Afterward sections were incubated with VECTASTAIN ABC Reagent (Vector Laboratories) and washed in PBS. Finally, sections were developed with peroxidase substrate solution (3,3'-Diaminobenzidine, DAB), dehydrated by successive ethanol (70%, 95%, 100%) and xylene baths and then mounted on Malinol mounting medium (MUTO PURE CHEMICALS CO.). Labelled areas were quantified in the neointimal tissue on digital images acquired at 40X magnification.

2.15. Statistical analysis

All quantified data represent the mean of at least triplicate samples. Error bars represent standard deviations of the mean. Statistical significance was determined using the Student's t-test, and $p < 0.05$ was considered significant.

3. Results

3.1. Effects of *Dendropanax morbifera* on cardiovascular diseases

3.1.1. Inhibitory effects on neointima formation

3.1.1.1. Anti-proliferative activities on RAoSMCs

First of all, to determine the working concentration, which is the highest concentration but may not cause the cell death, the indicated concentrations of the extracts of *D. morbifera* were tested the effects on cell viability using MTT assay. As shown in Fig. 1A, the extarcts of *D. morbifera* did not show any toxicity in RAoSMCs up to 50μg/ml. So the working concentration were determined with 25μg/ml in further study.

To investigate the anti-proliferative effects of extracts from *D. morbifera* on RAoSMCs, BrdU assay were performed in cells treated with the designated concentrations (Fig. 1B). The proliferative activities were reduced in concentration-dependent manner and were significantly decreased by ~40% in RAoSMCs treated 25μg/ml of *D. morbifera*.

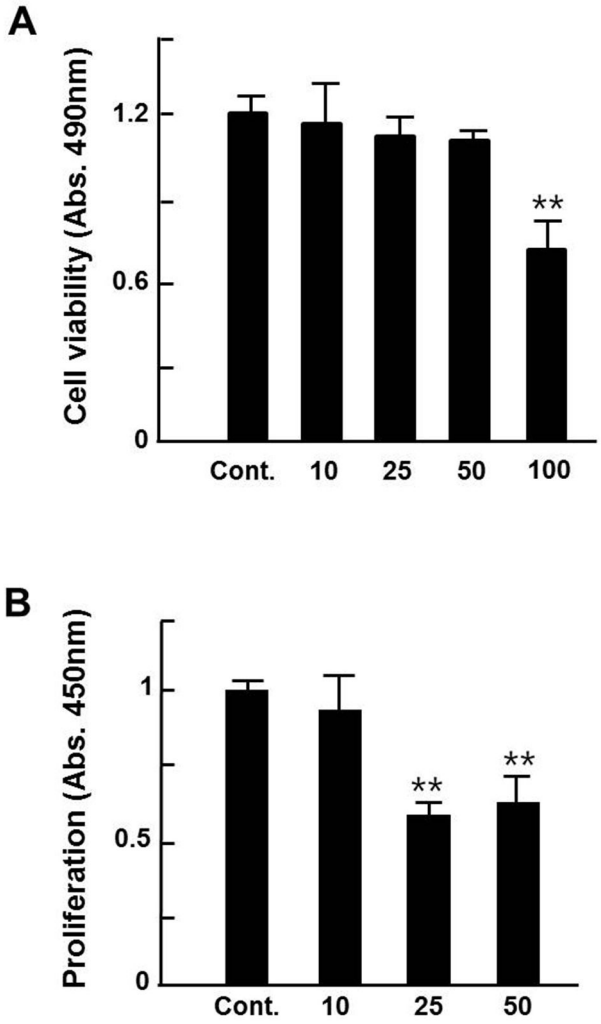


Figure 1. Cytotoxicity and anti-proliferative activities of the extracts from *D. morbifera* on RAoSMCs. (A) RAoSMCs were treated with the extracts from *D. morbifera* at the indicated concentrations for 24h. Cell viability was measured using the MTT assay. Results are expressed as cell viability relative to untreated controls. (B) The effects of the extracts from *D. morbifera* on RAoSMCs proliferation measured by BrdU assay. Values are presented as mean±S.D. **P<0.01 vs control.

3.1.1.2. Inhibitory effects of the extracts from *D.morbifera* on RAoSMCs migration

VSMC migration also significantly contributes to vascular disease development. Thus, to investigate whether the extracts from *D. morbifera* affects RAoSMCs migration, two-dimensional wound healing and three-dimensional Boyden chamber cell migration assays were conducted. As shown in Fig. 2A, the migratory distances in 2-D migration assay were significantly decreased by more than 40% in RAoSMCs compared to control. To confirm this result, a three-dimensional migration assays using transwell chambers were performed. As shown in Fig. 2A, the migrated cells through membranes of RAoSMCs were significantly reduced by ~50% compared to control cells. The quantitative analyses were performed in Fig. 2B.

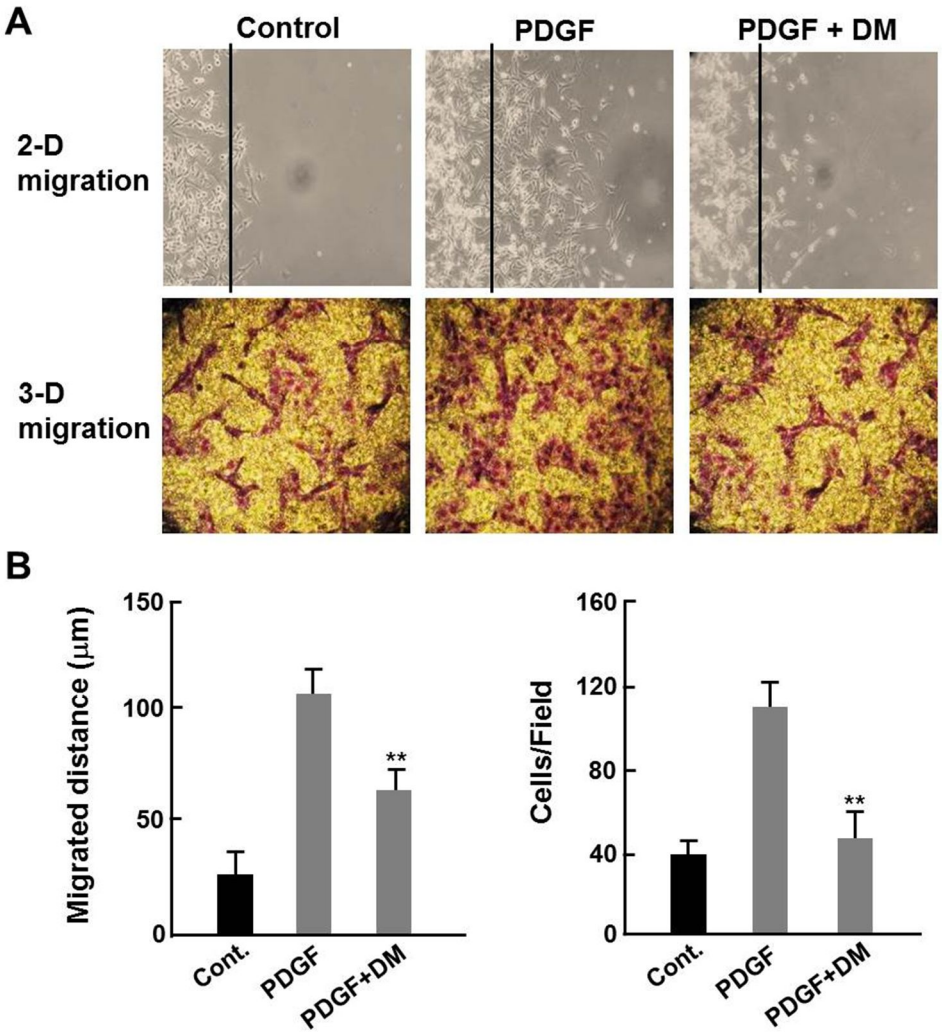


Figure 2. Effect of *D. morbifera* on RAoSMCs migration. Effects of extracts using various methods from *D.morbifera* on RAoSMCs migration. Migration rate were determined by both 2-D wound healing assay (A)-up and 3-D Boyden chamber assay (A)-down. Rat aortic smooth muscle cells (RAoSMCs) were treated with the designated concentration of the extracts determined in the Fig 1. Values are presented as mean±S.D. $^{**}P < 0.01$ vs control.

3.1.1.3. Altered mRNA levels of MMP2 and 9 by the extracts from *D. morbifera* in RAoSMCs

Since it is well known that MMP2 and MMP9 are key regulators of VSMCs migration in artherogenesis [10, 85], the effects of extracts from *D. morbifera* on the levels of MMPs expression were investigated. Treatment of the extracts from *D. morbifera* significantly reduced the expression levels of MMP2 and MMP9 mRNA whereas the levels of MMP7 were not altered. These suggest that the extracts from *D. morbifera* have the inhibitory effects on RAoSMCs migration through the specific regulation of MMP2 and MMP9 expression.

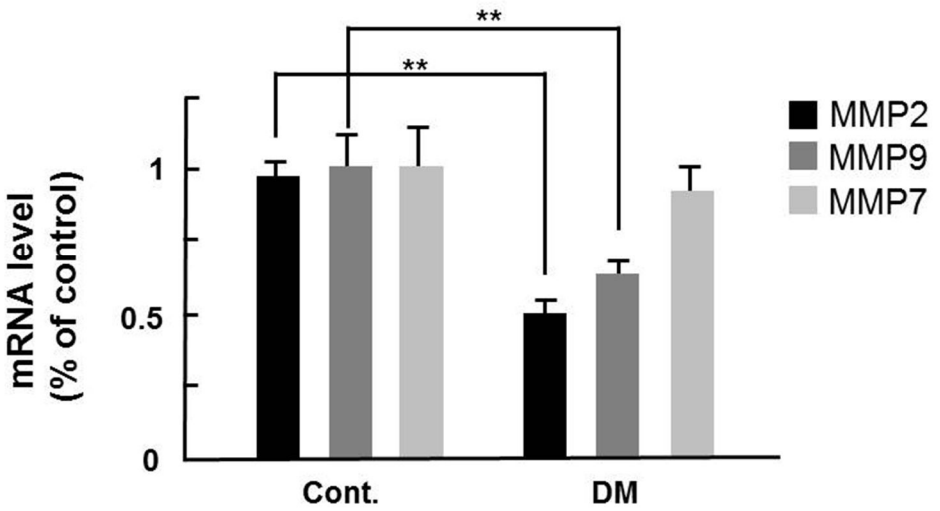


Figure 3. Effect of *D. morbifera* on MMPs expression in RAoSMCs. The MMP2, 7, and 9 mRNA levels were measured by RT-PCR. GAPDH was used as an internal control. Values are presented as mean±S.D. $^{**}P < 0.01$ vs control.

3.1.1.4. Altered expression of MMPs by the extracts from *D.morbifera* in RAoSMCs

Previous studies have shown that the PDGF induced expression of MMPs including MMP2 and MMP9 plays a pivotal role in RAoSMC migration via extracellular matrix (ECM) degradation, which is strongly linked to atherosclerotic development [86]. As shown in Fig. 4, western blot assay results through both the media and cell lysate showed that PDGF treatment of RAoSMCs substantially induced MMP2 and MMP9, 48h after PDGF exposure and *D. morbifera* was found to inhibit the PDGF induced MMP2 and MMP9 expression. These results indicate that *D. morbifera* attenuates PDGF induced RAoSMCs migration, as well as MMP2 and MMP9 expression which could play a pivotal role in ECM degradation and cell migration.

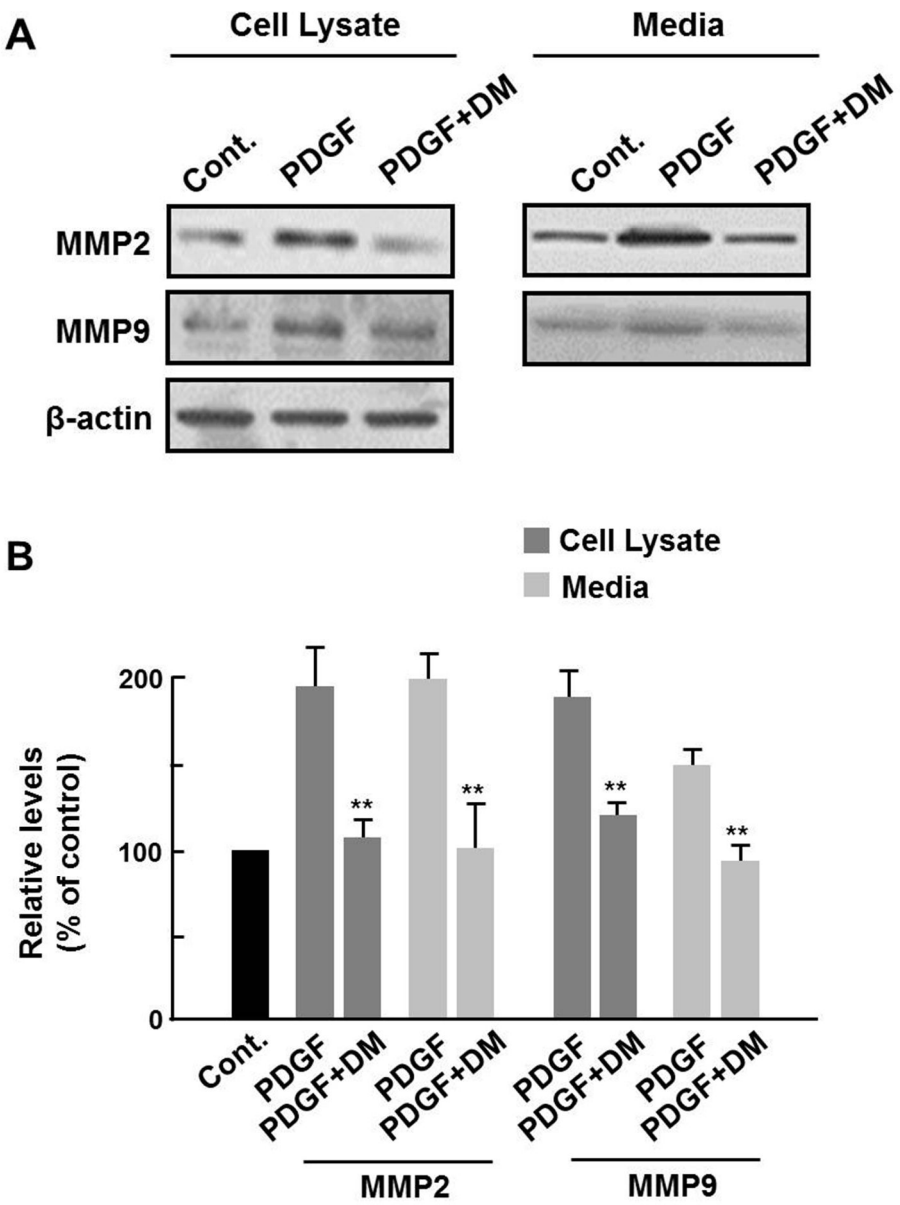


Figure 4. Effect of *D. morbifera* on MMP2 and MMP9 expression in RAoSMCs. (A) Western blot were conducted for the evaluation of the altered amount of MMP2 and MMP9 expression both in the media and cell lysate. (B) Altered expression levels of MMPs by DM in RAoSMCs showed the ratio of densities by the bar graphs. β-actin was used as a loading control. Values are presented as mean±S.D. $^{**}P < 0.01$ vs control.

3.1.1.5. Altered enzymatic activity of MMPs by the extracts from *D. morbifera* in RAoSMCs.

In order to assess the role of *D. morbifera* in the expression of MMP2, a gelatin zymographic assay was performed. Media from RAoSMCs induced by PDGF showed proteolytic activity at 72kDa, corresponding to MMP2. This induction of MMP2 expression by PDGF was suppressed following *D. morbifera* treatment. As showed in Fig. 5, similar results were observed with western blot analysis.

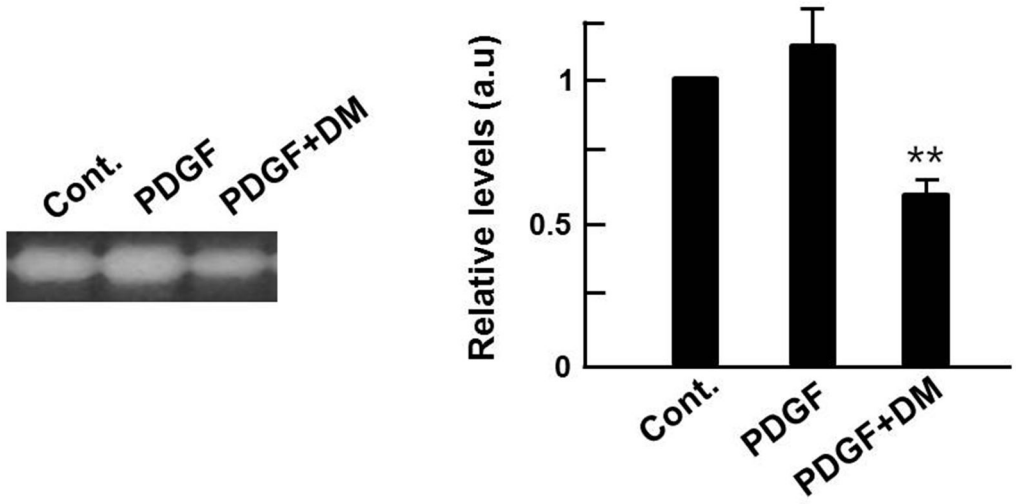


Figure 5. Effect of *D. morbifera* on MMPs enzymatic activity in RAoSMCs. The conditioned media were prepared and used for gelatin zymography. Values are presented as mean±S.D. ***P* <0.01 vs control.

3.1.1.6. Altered phosphorylation of signal mediators by the extracts from *D. morbifera* in RAoSMCs

The signal transduction pathways that modulate the activity of MMP transcription factors are various depending on cell types [87, 88]. MAPKs, which have different functions and can crosstalk at several levels to initiate the transcription of several immediate early genes required for proliferation, are activated in response to inflammatory and atherogenic stimuli including PDGF [18]. Serum deprived VSMCs were stimulated with PDGF for 30min and the activation of AKT and ERK 1/2 were determined by western blot. As shown in Fig. 6, stimulation with PDGF overexpressed p-AKT and p-ERK 1/2. But, the level of phosphorylated Akt and ERK were selectively decreased in *D. morbifera*-treated RAoSMCs. These results demonstrate that Akt and ERK are likely a downstream target of *D. morbifera*-mediated signaling that regulates MMP2 and MMP9 expression and their functions in cell migration in RAoSMCs.

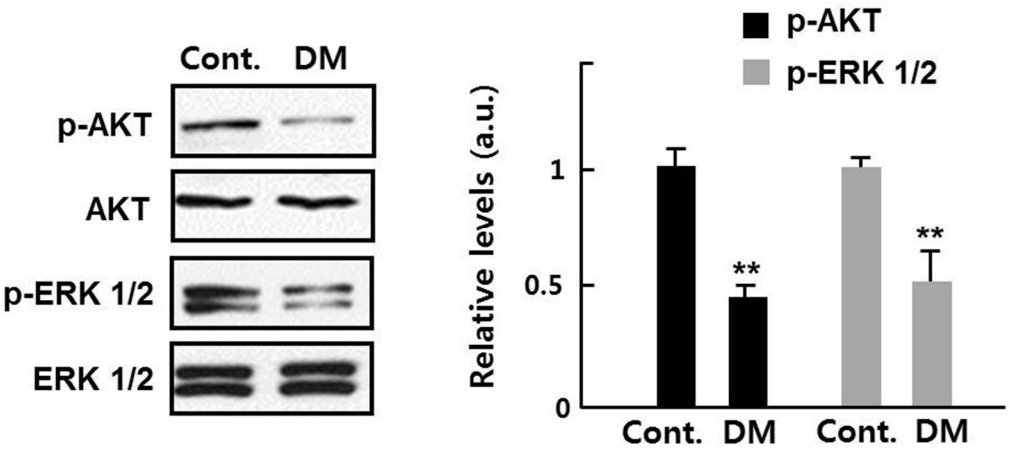


Figure 6. Effect of *D. morbifera* on phosphorylation of signal mediators in RAoSMCs. Serum starved RAoSMCs were pretreated with DM for 1h followed by stimulation with PDGF for additional 30min. The cell lysates were assayed for protein expression of p-AKT and p-ERK 1/2. Values are presented as mean±S.D. ***P* <0.01 vs control.

3.1.1.7. Inhibitory effects of the extract from *D. morbifera* on neointima formation of balloon injury rat model

To investigate whether DM also prevents restenosis, we established a balloon injury rat model, we established a balloon injury rat model, and neointimal hyperplasia in injured carotid arteries was estimated by calculating the intima to media ratio (I/M ratio) after measuring the neointima and media area. Typical light micrographs of carotid arteries from the control and the DM-treated animals at 14 days after balloon injury are shown in Fig.7A. Intimal hyperplasia formation was quantitatively recorded by a computer-assisted morphometric analysis. There were no intimal lesions observed in the uninjured left carotid arteries in both control and treated animals. The intimal layer of these uninjured carotid arteries only comprised of one endothelial cell layer on the internal elastic lamina and very little subendothelial tissues. Balloon-injured right carotid arteries harvested from all groups exhibited loss of endothelium and development of intimal hyperplasia. Therefore, the intima area in the DM-treated group was considerably decreased compared with the reference group, although there was no significant difference in the media area between the two groups. As shown in Fig.7B, the mean intimal area of the injured only group is 135,382 ±30,862 μm^2, and administration of DM caused a dose-dependent inhibition of intimal hyperplasia formation. The intimal area and intima/media area ratio in the 90mg/kg/d group were decreased by 55.97%. The medial area in the right balloon-injured arteries and the left uninjured arteries was not significantly different.

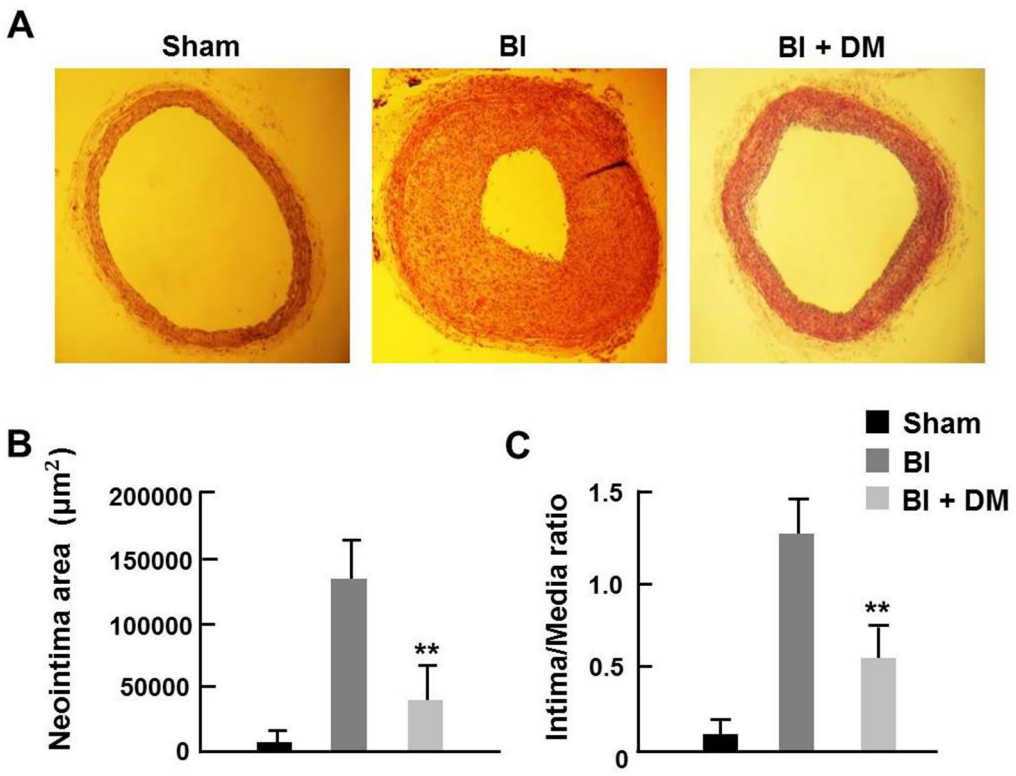
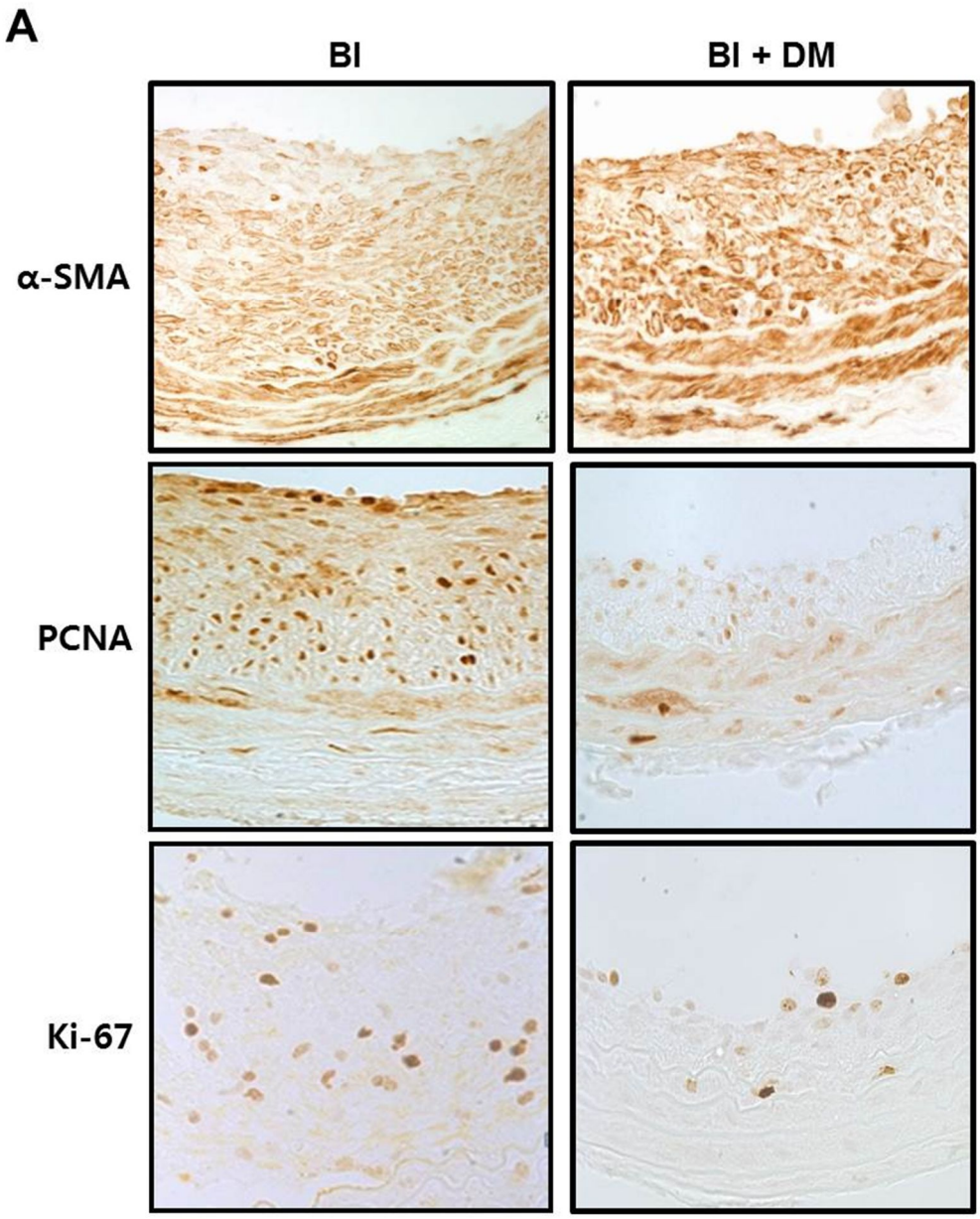


Figure 7. Effect of *D. morbifera* on neointimal hyperplasia in balloon-injured rat carotid arteries. Rats were orally administered *D. morbifera* (90mg/kg/d) for two weeks after balloon injury. (A) Neointima formation is shown in a representative cross-section of the common carotid artery by hematoxylin-eosin staining by 100x magnification. (B) The degree of restenosis was estimated by calculating the morphologic analysis of neointima area and intima/media ratio. Values are presented as mean±S.D. ***P* <0.01 vs control.

3.1.1.8. Inhibitory effects of the extract from *D.morbifera* on intimal cell proliferation and migration

At 14 days after injury, proliferation and migrating cells in the balloon-injured arteries were readily detected. Balloon-injured rat carotid arteries in injury group revealed strong positive signals for α-SMA, PCNA and Ki-67, which are proliferation protein markers, while those from the DM-treated group reduced the positive signals. Furthermore, Balloon-injured rat carotid arteries in injury group revealed strong positive signals for MMP2 and MMP9, which are migration protein markers. However, MMP2 and MMP9 is significantly less expressed in the *D. morbifera*-treated groups. Thus, *D. morbifera* significantly reduced the positive signals in neointima, consistent with histopathological changes in neointima. Representative immunohistochemical stains are shown in Fig. 8.



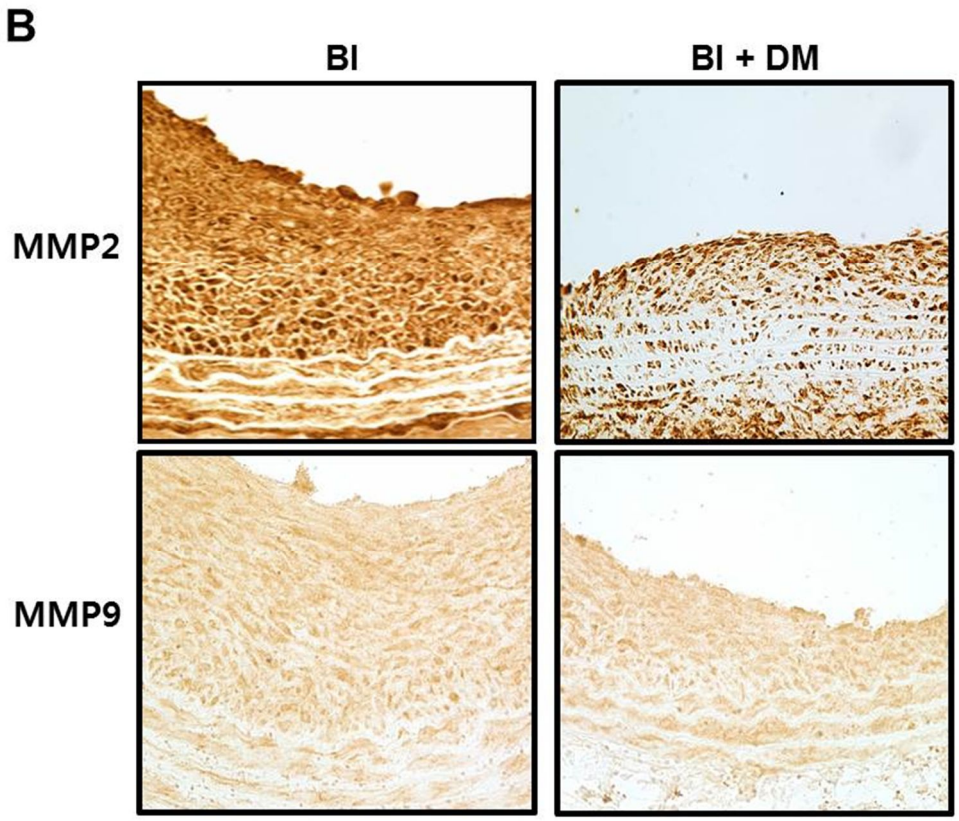


Figure 8. Effect of *D. morbifera* on cell proliferation and migration in balloon-injured rat carotid arteries. Tissue sections of balloon-injured rat carotid arteries from the injured group and *D. morbifera*-treated group were subjected to immunohistochemistry with anti α-SMA, PCNA, Ki-67 (A), MMP2 and MMP9 antibodies (B), which are both specific antigen markers for cell proliferation and migration (magnification, 40x).

3.1.1.9. Altered expression of proteins associated with neointima formation

MMP2 and PCNA expression levels by western blot were assessed in neointimal lesions to determine the inhibitory effects of *D. morbifera* on migration and proliferation. Both proteins were suppressed in the *D. morbifera*-treated group, while those from the balloon-injured group were highly expressed (Fig. 9A). In addition, as shown in Fig. 9B, the mRNA levels of MMP2 and PCNA were up-regulated in the balloon-injured group, but were down-regulated in the *D. morbifera*-treated group. These results suggested that VSMC proliferation and migration are regulated *in vivo* by DM after balloon injury.

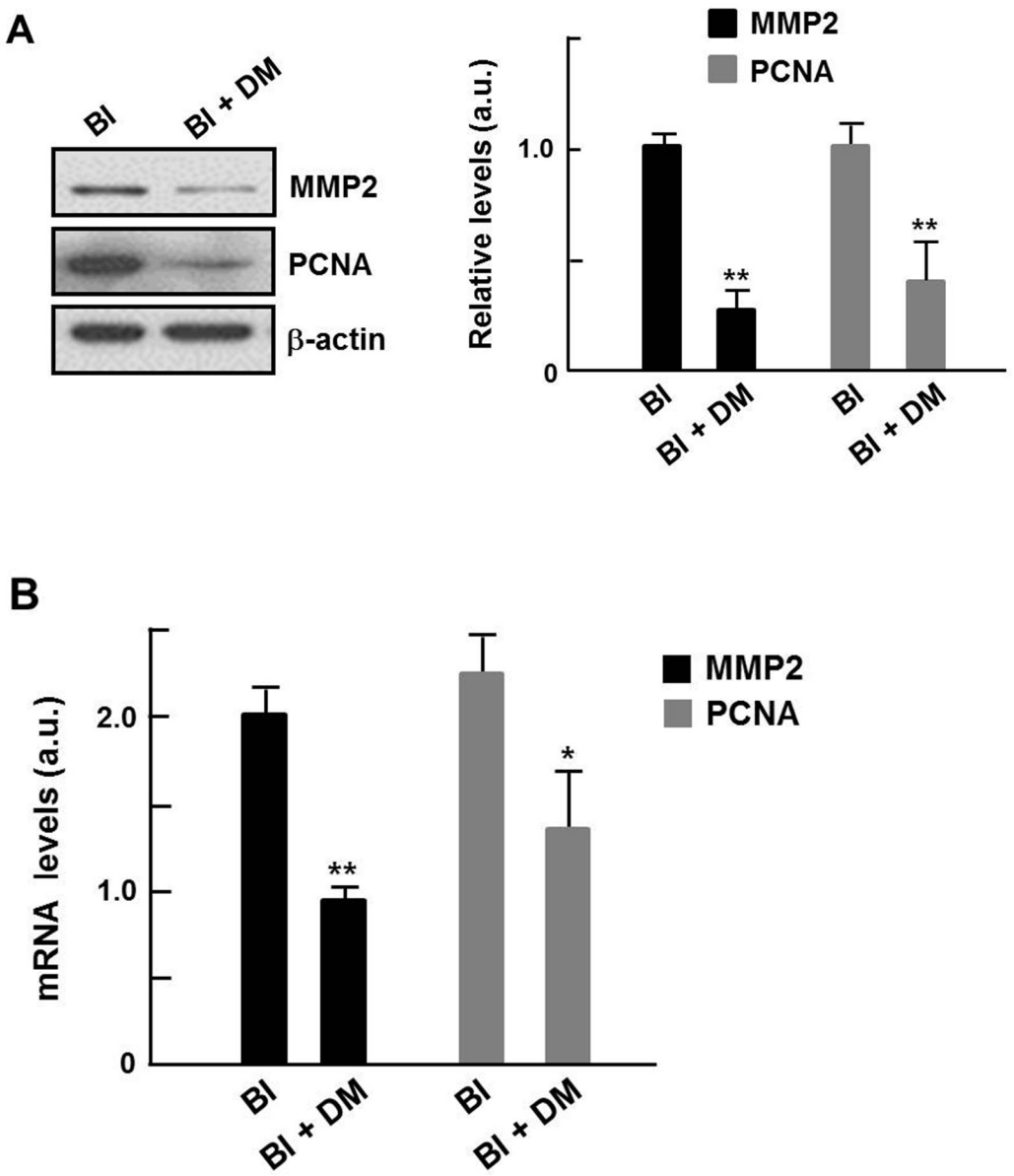


Figure 9. Effects of *D. morbifera* on MMP2 or PCNA protein expression in *in vivo*. (A) The expression of MMP2 and PCNA were detected by western blot. (B) mRNA expression was analyzed by real-time RT-PCR. Values are presented as mean±S.D. $^{**}P < 0.01$ vs control.

3.1.2. Protective effects on myocardial injury

3.1.2.1. Anti-apoptotic effects in hypoxia-reoxygenated cardiomyocytes

In the last decade, distinct modes of cell injury in response to ischemia-reperfusion have been described [89]. Indeed, it has been demonstrated that reperfusion of previously ischemic tissues leads to an additional injury beyond that caused by ischemia alone [90, 91]. Many evidences demonstrated that hypoxia alone causes time dependent apoptosis, and reoxygenation increases the number of apoptotic cells beyond that in response to hypoxia alone. In this study, the effects of the extracts of *D. morbifera* on hypoxia-reoxygenation injured cardiomyocytes (CMC) were investigated. As shown in Fig.10, the condition of cardiomyocytes with hypoxia (30min) and reoxgenation (1hr) decreased the cell number, but the treatment of *D. morbifera* inhibited CMC death induced apoptosis. To confirm that the inhibitory effects were not due to toxicity or damage to the cells, various concentrations of *D. morbifera* were treated in nonstimulated cells for 24h. *D. morbifera* at a concentration of 25 μg/ml had no effect on the basal level of cell viability (data not shown). These results suggested that the progress of apoptosis in CMC are regulated by *D. morbifera* after hypoxia/ reoxygenation.

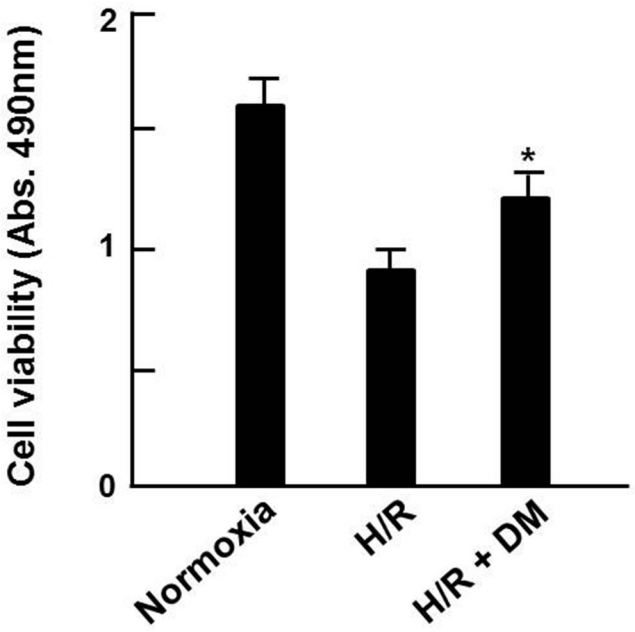
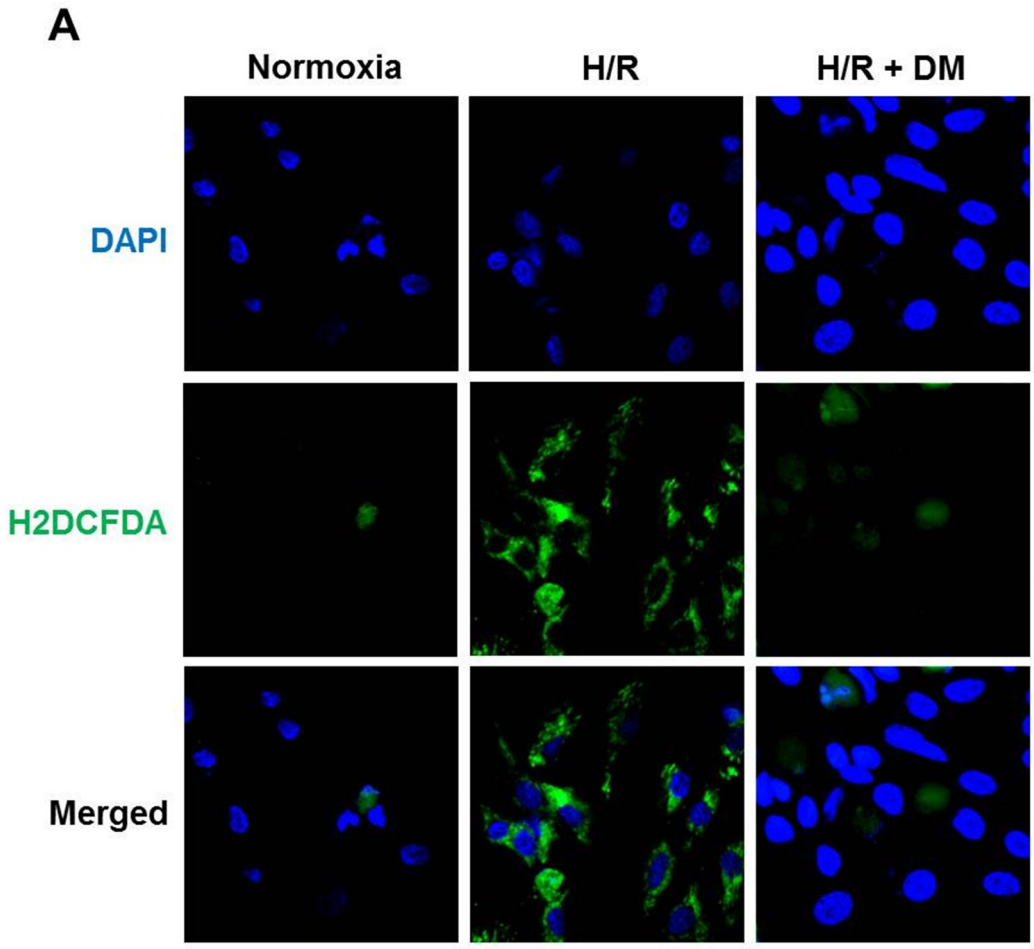


Figure 10. Protective effects of *D. morbifera* on cardiomyocytes injured by hypoxia/reoxygenation. Summary of the data on hypoxia (30min)-reoxygenation (1hr) induced apoptosis and the effect of *D. morbifera* by MTT assay. Values are presented as mean±S.D. **P* <0.05 vs control.

3.1.2.2. Altered generation of intracellular ROS by the extracts from *D. morbifera* in cardiomyocytes

Several studies have demonstrated that the production of ROS are an important role in vascular inflammation and restenosis [92]. Therefore, to confirm whether the *D. morbifera* has inhibitory effect of intracellular ROS generation in CMC, *D. morbifera* was treated in condition of hypoxia-reoxygenation and used the principle of measuring ROS. 2',7'-dichlorofluorescein diacetate (DCFH-DA) is a cell-permeable non-fluorescent probe that is de-esterified intracellularly and rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. As shown in Fig. 11, *D. morbifera* treated CMC reduced ROS generation compared with positive control (the condition of hypoxia-reoxygenation). These results demonstrate that *D. morbifera* protect CMC of hypoxia-reoxygenation state through decreased ROS generation.



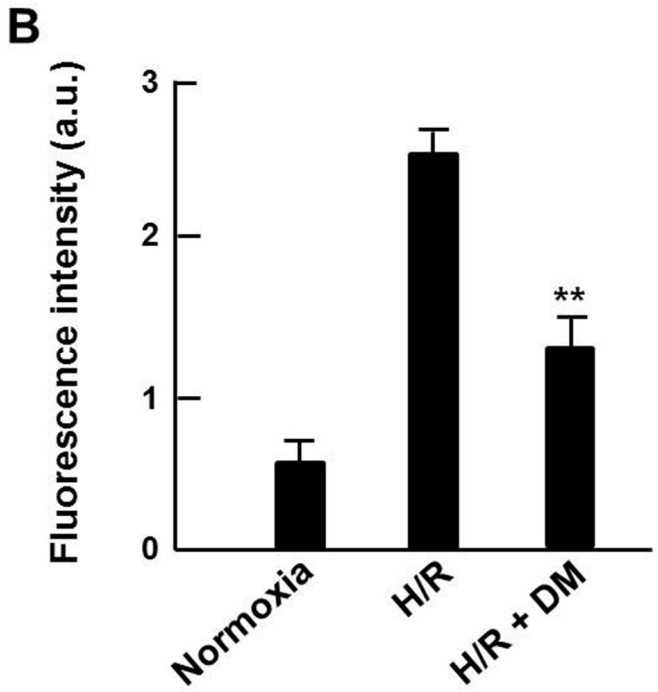
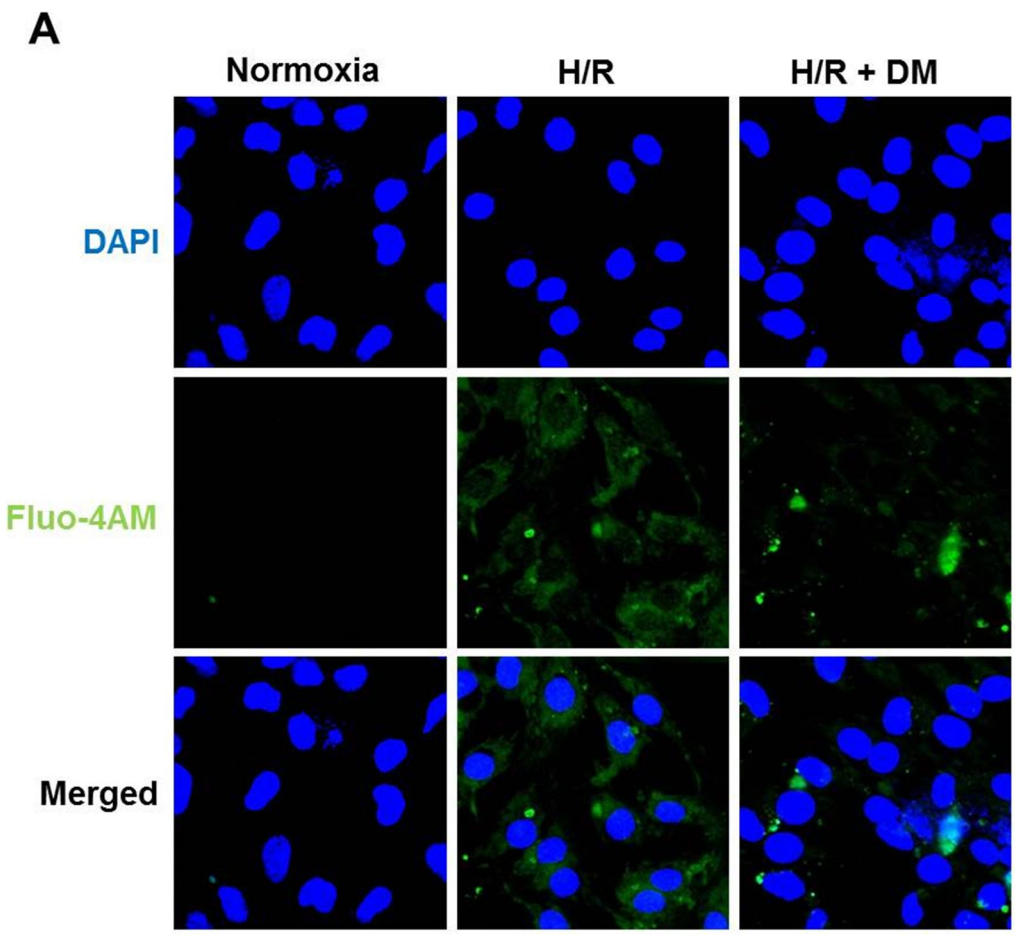


Figure 11. Inhibitory effect of *D. morbifera* on intracellular ROS generation in cardiomyocytes. Evaluation of intracellular peroxide generation was based on the dye 2',7'-dichlorofluorescein diacetate (H_2DCF-DA) the fluorescence. (A) Negative control (DMSO), Positive control (the state of hypoxia-30min and reoxygenation-1hr), DM (pretreatment 1hr before the state of hypoxia-30min and reoxygenation-1hr), intracellular H_2DCF-DA images were taken by confocal laser microscope (Olympus, Japan) using excitation and emission wavelengths of 488 nm and 520 nm respectively (fluorescent images were magnified ×400). (B) The fluorescence intensity of an equivalent field size (2.5 × 2.5 mm) was measured using the Image J quantification software. Values are presented as mean±S.D. $^{**}P$ <0.01 vs control.

3.1.2.3. Intracellular calcium handling by the extracts from *D. morbifera* in cardiomyocyte

Intracellular calcium (Ca(2+)i) overload induced by chronic hypoxia alters Ca(2+)i homeostasis, which plays an important role on mediating myocardial injury [93]. To confirm whether treatment with DM improve Ca^{2+} handling in hypoxic myocardial injury, intracellular calcium concentration ([Ca(2+)]i) was measured with Fluo-4/AM. As shown in Fig.12, the cardiomyocyte were alleviated in hypoxic cardiomyocyte treated with DM compared with hypoxic cardiomyocyte. These results suggest that DM ameliorates Ca(2+)i homeostasis, thereby producing the cardioprotective effect and reduction in hypoxic cardiomyocytes damage.



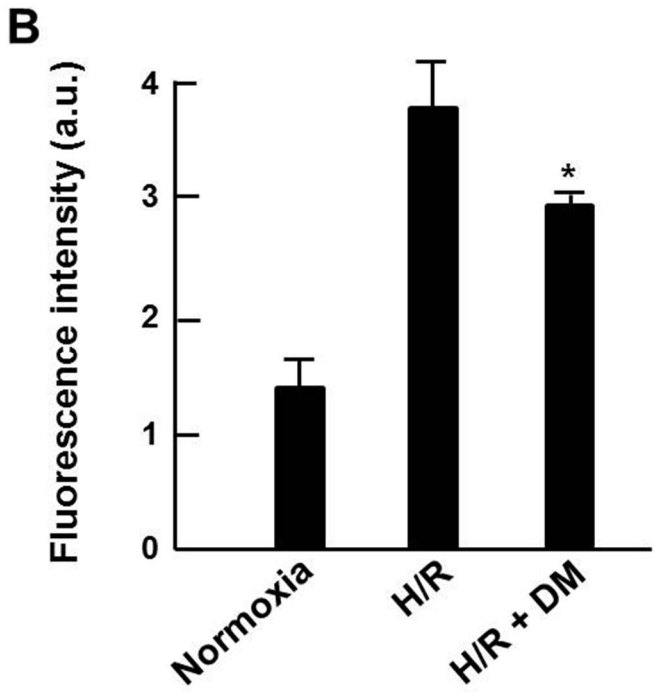


Figure 12. Effects of *D. morbifera* on intracellular calcium handling in cardiomyocytes.
(A) Cardiomyocytes were exposed to Negative control (DMSO), Positive control (the state of hypoxia-30min and reoxygenation-1hr), DM (pretreatment 1hr before the state of hypoxia-30min and reoxygenation-1hr), Intracellular calcium fluorescence was measured Fluo-4am. intracellular Fluo-4am images were taken by confocal laser microscope (Olympus, Japan) using excitation and emission wavelengths of 488 nm and 520 nm respectively (fluorescent images were magnified ×40). (B) The fluorescence intensity of an equivalent field size (2.5 × 2.5 mm) was measured using the Image J quantification software. Values are presented as mean±S.D. **P* <0.05 vs control.

3.1.2.4. Altered expression levels of calcium homeostasis-related proteins by the extracts from *D. morbifera* in cardiomyocytes

Induced defective intracellular Ca2+([Ca2+]i) homeostasis and increased reactive oxygen species (ROS) production by chronic hypoxia which plays an important role on mediating myocardial injury [94, 95]. Several ion transport pathway are highly sensitive to redox regulation and oxidative stress directly impedes intracellular Ca2+ homeostasis [96]. Downregulation of key Ca2+-handling proteins like sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA)2a and ryanodine receptor (RyR2) is one of the major cause of abnormal Ca2+ homeostasis in cardiomyopathy [97-99]. The alteration in SERCA2a and RyR2 expression results in altered cytosolic Ca2+ transients, leading to abnormal contraction [100]. Also recently, it was revealed that oxidative stress can activate calcium calmodulin kinase Ⅱ (CaMK Ⅱ) [101]. Therefore, altered expression levels of calcium homeostasis-related proteins by DM in cardiomyocytes performed by western blot. In DM-treated cardiomyocytes exposured in hypoxia, we observed a decrease in expression of ryanodine receptor 2 (RyR2) and phosphorylated Ca(2+)/calmodulin-dependent protein kinase Ⅱ(p-CaMK Ⅱ) and enhanced expression of sarco(endo)-plasmic reticulum Ca(2+)-ATPase (SERCA2a). DM attenuated abnormal changes of RyR2 and SERCA2a genes in hypoxic cardiomyocytes. These findings indicate that DM treatment under myocardial injury condition prevents dysregulation of calcium regulatory proteins in the heart.

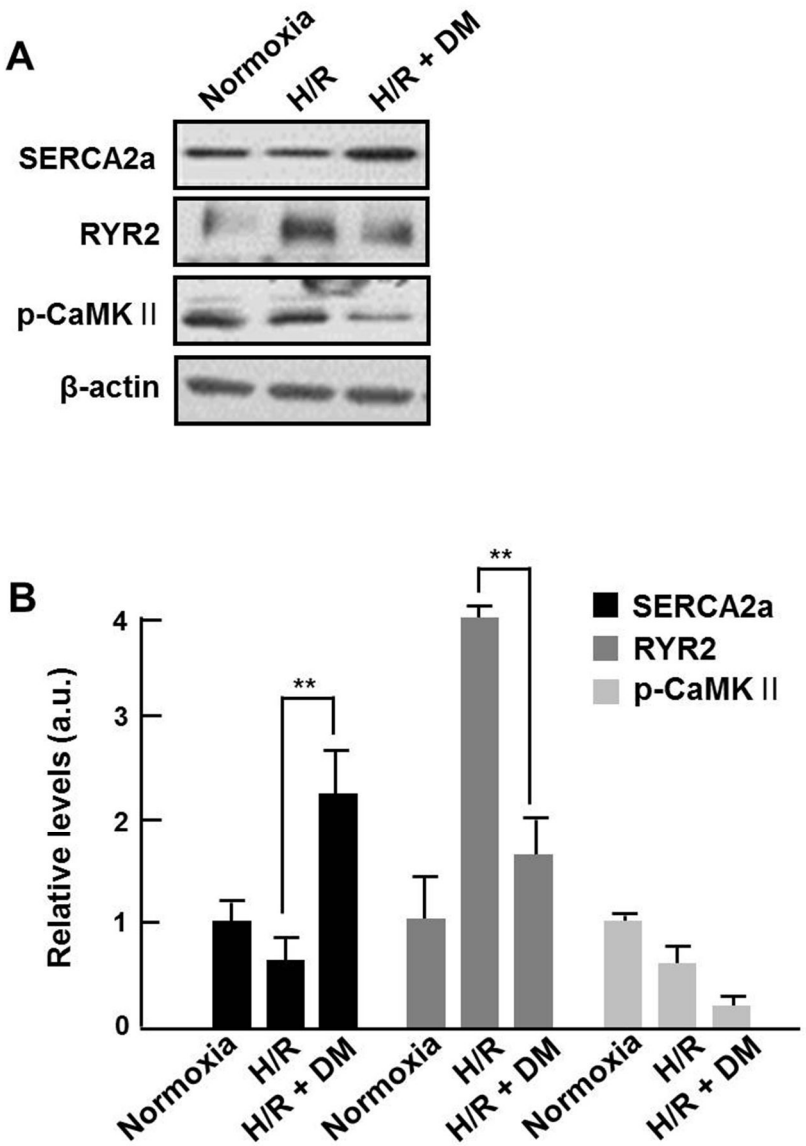


Figure 13. Altered expression levels of calcium homeostasis-related proteins by *D. morbifera*. (A) SERCA2a, p-CaMKII and RyR2 expression were measured by western blot. Rat cardiomyocytes were treated for 24 hours with DM, phospholyation level were treated for 30min. protein-band intensities normalized to ß-actin, relative to control. (B) Protein-band intensities were normalized to SERCA2a, p-CaMKII and RyR2, relative to control. Values are presented as mean±S.D. ***P* <0.01 vs control.

3.2. Additional natural products on cardiovascular diseases

3.2.1. Anti-proliferative effects of the extracts

In order to screen the natural products having activities for cardiovascular diseases, 31 more extracts besides *D. morbifera* were tested. Candidates were chosen through a literature research and No. 1~23 were obtained from Korea plant extract bank and 24~31 were made by this laboratory followed by the method described in Methods section (Table 2).

First of all, the working concentrations of each extracts were determined by the highest concentration showing no cytotoxicity (Fig. 14A). Then, each extracts were tested whether they have anti-proliferative activities on RAoSMCs. Interestingly, the extracts from *Pinus densiflora* have shown significant inhibitory effects, regardless of what solvent were used, on proliferation of RAoSMCs compared to control (Fig. 14B). It has shown the dramatic decrease of proliferation, which was similar or even lower than Epigallocathechin-3-gallate (EGCG)-treated RAoSMCs. EGCG was used positive control because it has been well known to have anti-proliferative activity on VSMCs.

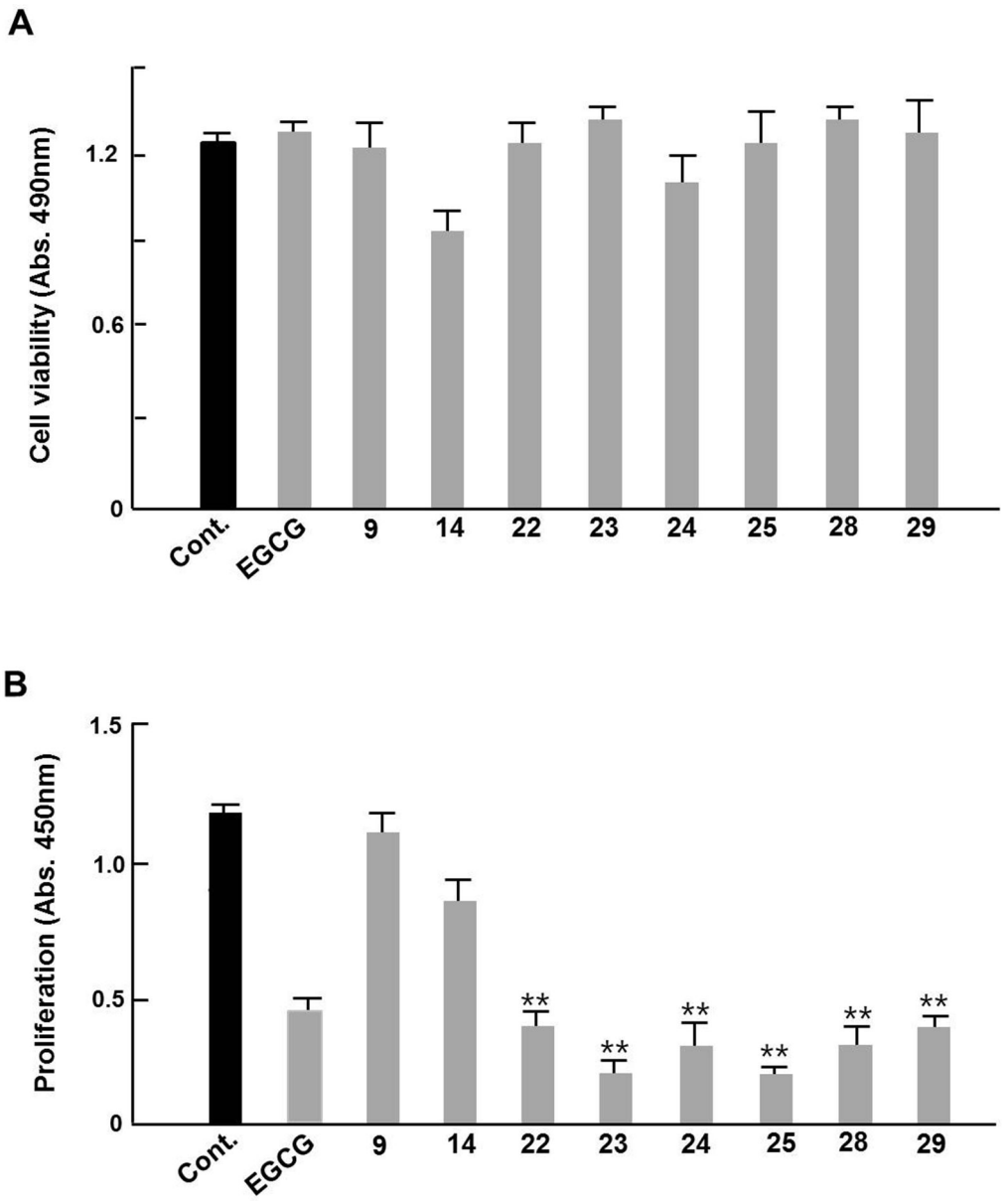
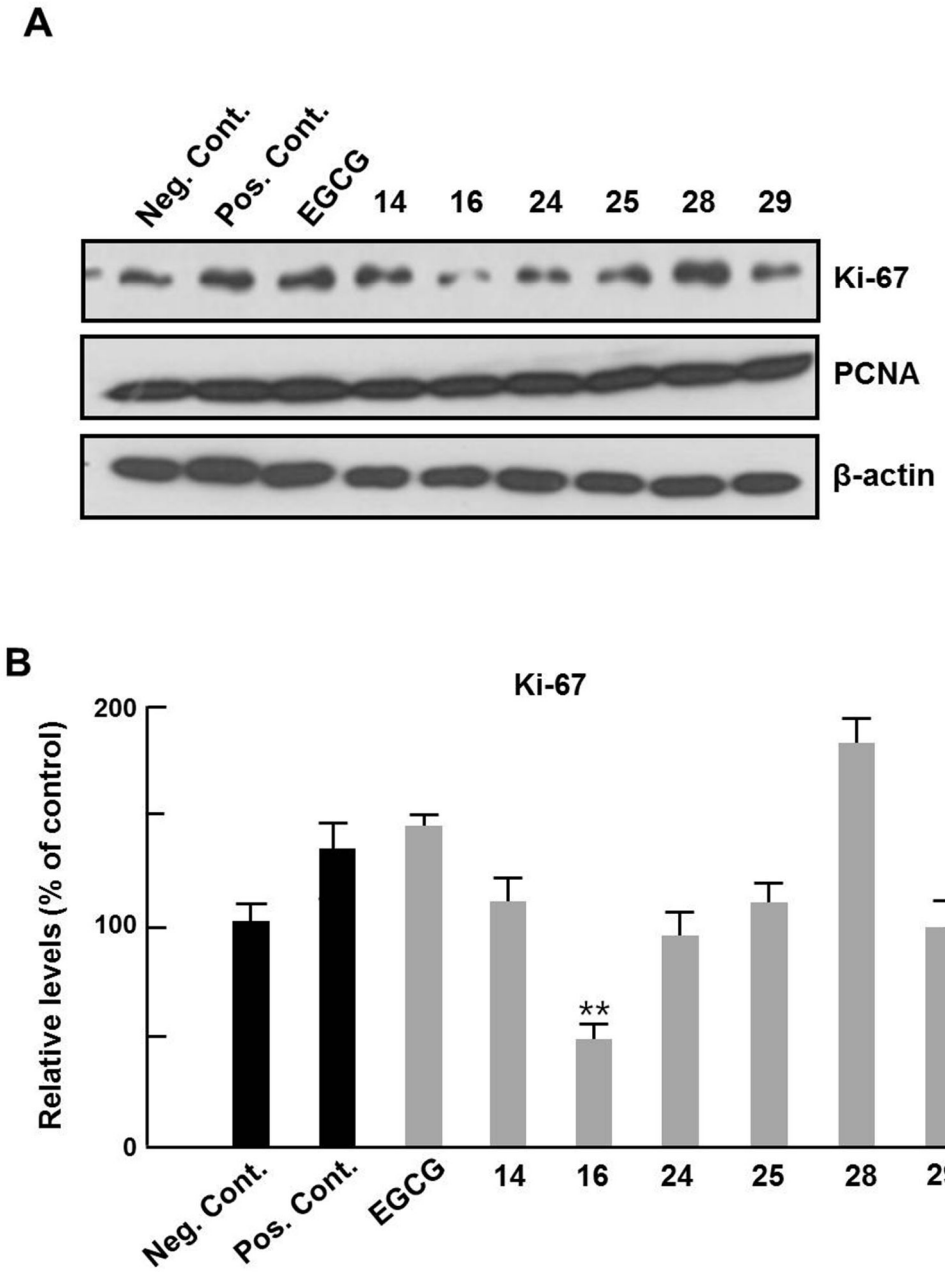


Figure 14. Anti-proliferative effects of the various extracts on RAoSMCs. (A) Cytotoxic effects of the various extracts acquired from Korea plant extract bank on RAoSMCs using MTT assay, (B) Inhibitory effects of the various extracts acquired from Korea plant extract bank on RAoSMCs proliferation using BrdU assay.

3.2.2. Altered protein levels of Ki-67, PCNA, and MMP2 by the extracts from *Plantago asiatica* and *Pinus densiflora*

Further, western blot analysis were performed whether the expression levels of proliferation or migration associated proteins were altered. As shown in Fig 15A, the expression levels of the proliferating marker Ki-67 was significantly decreased in *P. asiatica*-treated RAoSMCs compared to control, which was lower than EGCG-treated cells (Fig. 15A). However, the level of PCNA was not altered, indicating that the anti-proliferative effect of *P. asiatica* is through the cell cycle specific regulation. In addition, even the degree of inhibition was lower than those of *P. asiatica*, the extract of *P. densiflora* decreased the level of Ki-67 (Fig. 15A). Interestingly, the extracts from *P. asiatica* have shown no activities on the level of MMP2 expression whereas the extracts from *P. densiflora* have decreased significantly the expression level of MMP2 (Fig. 15B). These results indicated that the activity of *P. asiatica* is specific on anti-proliferation and those of *P. densiflora* is specific on anti-migration in RAoSMCs. Further studies will be needed to reveal the mechanisms underlying the effects of the extracts on the regulation of RAoSMCs.



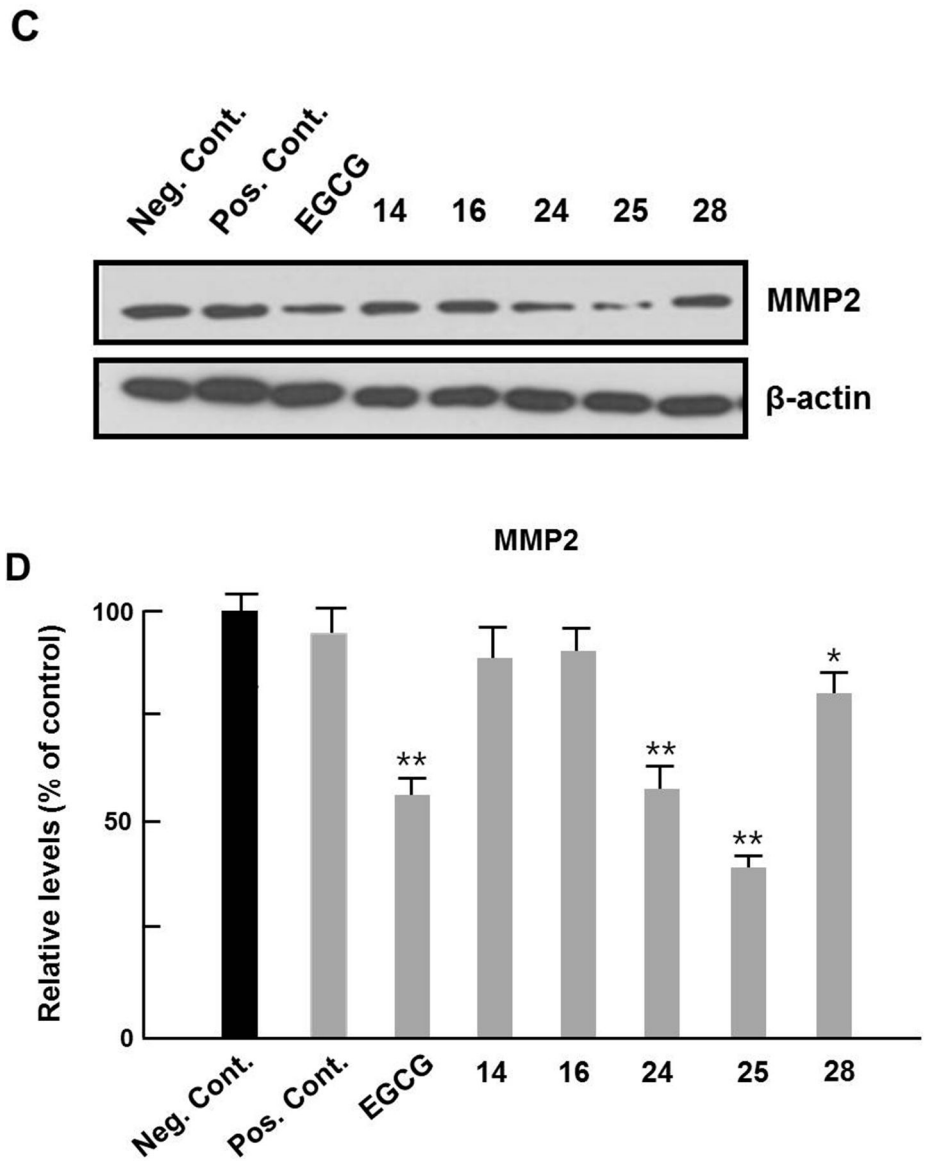


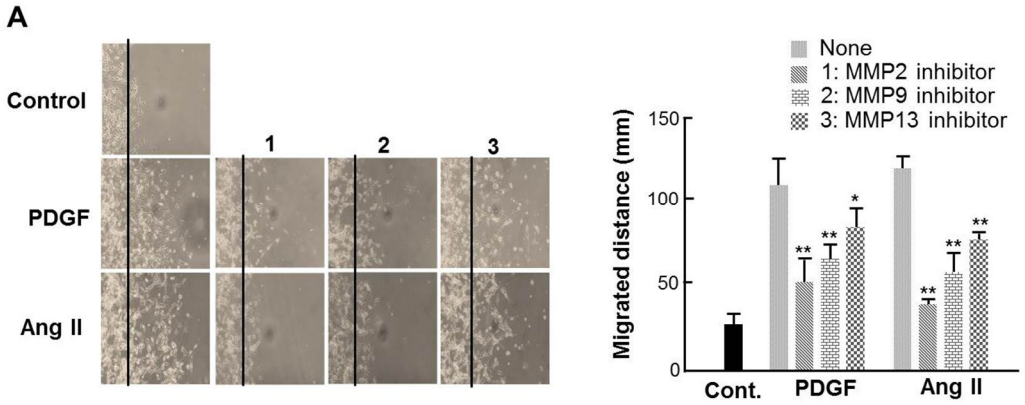
Figure 15. Altered protein levels of Ki-67, PCNA and MMP2 by the extracts from *Plantago asiatica*. (A) The expression of Ki-67, PCNA and (C) were measured by western blot. RAoSMCs were treated for 24hr with the extracts from *Plantago asiatica* and *Pinus densiflora.* (B) Protein-band intensities normalized to Ki-67 relative to control. (D) Protein-band intensities normalized to MMP2 relative to control.

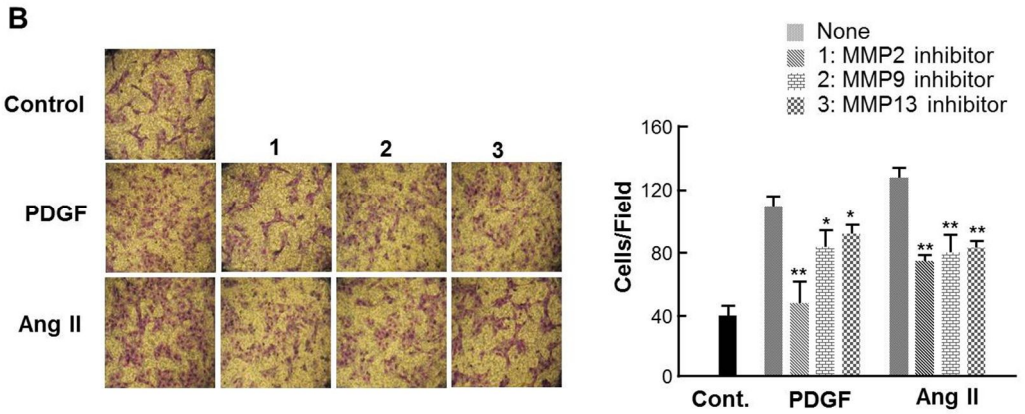
3.3. MMP13 is an additional regulator of VSMCs migration.

3.3.1. Inhibition of MMP13 activity results in a reduction of PDGF- or Ang II-induced RAoSMCs migration

We confirmed that treatment of RAoSMCs with PDGF or Ang II increased RAoSMCs migration in a dose-dependent manner using both two-dimensional wound healing and three-dimensional Boy-den chamber assays (data not shown). Fig. 16 shows that 5 ng/ml PDGF or 200 nM Ang II increased RAoSMCs migration significantly in both two- and three-dimensional assays. Because MMP2 and MMP9 are major mediatorsof RAoSMCs migration, we compared the effects of MMP activity inhibition using specific inhibitors on RAoSMCs migration. As expected, treatment with an MMP2-specific inhibitor decreased both PDGF- and Ang II-induced two-dimensional RAoSMCs migration by ～50% and ～70%, respectively, compared with that in untreated controls. Treatment with an MMP9-specific inhibitor also showed a significant reduction in PDGF- or Ang II-induced RAoSMCs migration. Interestingly, treatment with an MMP13-specific inhibitor resulted in significant reductions of both PDGF- and Ang II-induced two-dimensional RAoSMCs migration by ～20%and ～30%, respectively (Fig. 16A), even though the degree of reduction was lower than those of MMP2 and MMP9. We also performed three-dimensional Boyden chamber assays to confirm these results. As shown in Fig. 16B, treatment with the MMP13-specific inhibitor reduced both PDGF- and Ang II-induced RAoSMCs migration by ～20% and ～30%, respectively, which were almost the same as those in the two-dimensional assay. In particular, the reduction of Ang II-induced

RAoSMCs migration by MMP2, 9, or 13 was almost the same as that in the three-dimensional assay. Furthermore, to investigate the role of MMP13 in MMP2- and MMP9-mediated RAoSMCs migration, we evaluated the effects of co-inhibition of MMP13 activity with MMP2 or MMP9 activities on RAoSMCs migration using the three-dimensional Boyden chamber assay. As shown in Fig. 16C, no significant synergistic effect was observed by co-inhibition of MMP2 and MMP13 activities in PDGF-induced RAoSMCs migration compared with that of the MMP2inhibitor alone. However, we observed a significant reduction by co-inhibition of MMP9 and MMP13 activities in PDGF-induced RAoSMCs migration compared with that of the MMP9 inhibitor alone. We also observed obvious synergistic effects of co-inhibition in Ang II-induced RAoSMCs migration. Co-inhibition with MMP13 showed a ~20 % increase in the reduction of AngII-induced cell migration compared with that of the MMP2 or MMP9 inhibitors alone.





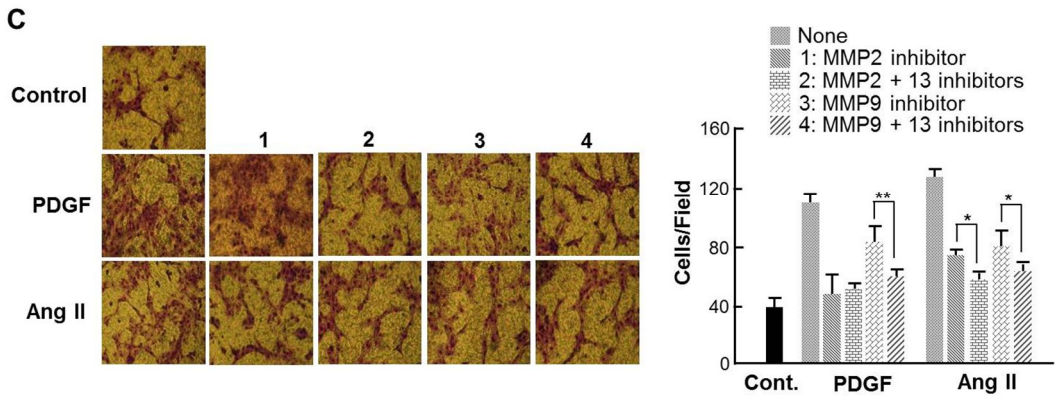
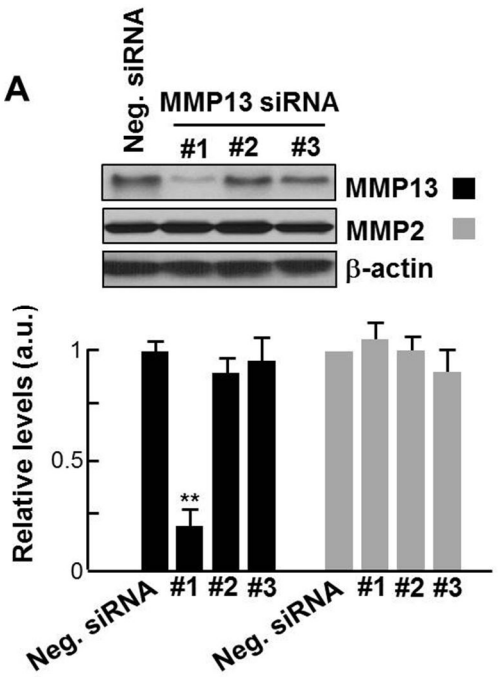


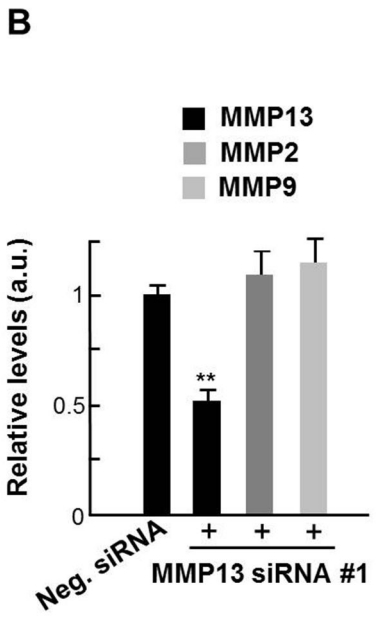
Figure 16. Effect of MMP13 inhibition on RAoSMCs migration. (A) Two-dimensional migration assays were conducted with RAoSMCs using a modified wound healing assay in the presence of 5 mM OA-Hy, 10 nM inhibitor I, or 20 nM pyrimidine dicarboxamide. The migrated distance during the designated period was measured. Scale bar, 200 ㎛. (B) Three-dimensional migration assays were performed with RAoSMCs using Boyden chambers in the presence of designated MMP inhibitors above. (C) Three-dimensional migration assays were conducted in co-treatment of pyrimidine dicarboxamide with OA-Hy or inhibitor I in RAoSMCs. Columns, average of triple determinations; bars, SD.*P < 0.05 or **P < 0.01 as compared with control.

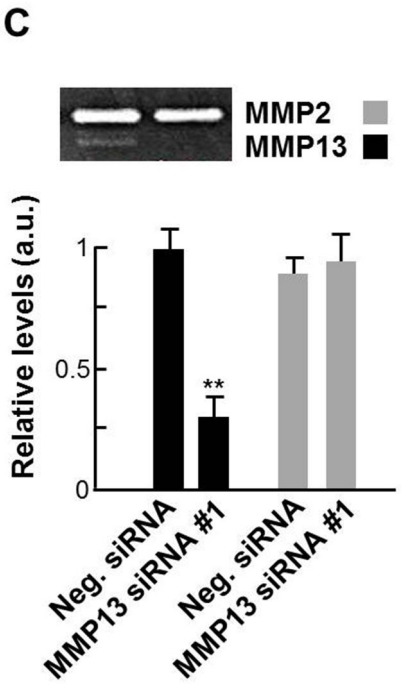
3.3.2. MMP13 silencing decreases PDGF- and AngⅡ-induced RAoSMCs migration

To determine the specific role of MMP13 in RAoSMCs migration, we knocked down MMP13 expression using an MMP13-specific siRNA. We designed three double-stranded, 21-nucleotide siRNAs with 30 TT dinucleotide overhangs against the coding sequence of rat MMP13 (NM 133530). None of the siRNAs shared homology with exons of other known rat genes. The three siRNAs were screened for their effect on MMP13 protein levels using Western blot in order to indicate the most effective silencer for further analysis. As shown in Fig. 17A, MMP13 siRNAs #2 and #3 had no significant effect on MMP13 expression. However, MMP13 siRNA #1 silenced Ang II-induced MMP13 expression by more than 80%, suggesting that MMP13 siRNA #1 could modulate MMP13 expression. The reduction in MMP13 expression levels was maintained up to 72 h after transfection with MMP13 siRNA #1 (data not shown). We confirmed that mRNA expression level of MMP13 was also significantly decreased in MMP13 siRNA-treated RAoSMCs (Fig. 17B). Addition-ally, we demonstrated the specificity of siRNA #1 for MMP13 by showing no cross-reactivity with MMP2 and 9 (Fig. 17A and B). We further observed that MMP13 secreted into media was significantly decreased by ~50% in zymographic analysis (Fig. 17C). To investigate whether MMP13 silencing affected RAoSMCs migration, we conducted three-dimensional cell migration assays using transwell chambers. We found that knockdown of MMP13in PDGF- or Ang II-treated RAoSMCs decreased cell migration by~40% and ~20%, respectively, compared with that in negative siRNA-treated controls (Fig. 17D). In addition, we examined the effect of MMP13 silencing on the survival and proliferative activity of RAoSMCs, because a decrease in survival or

proliferation after MMP13 silencing may have contributed to the observed decrease in RAoSMCs migration. We found that MMP13 silencing did not significantly decrease the proliferation rate of RAoSMCs compared with that of control cells (Fig. 17E).







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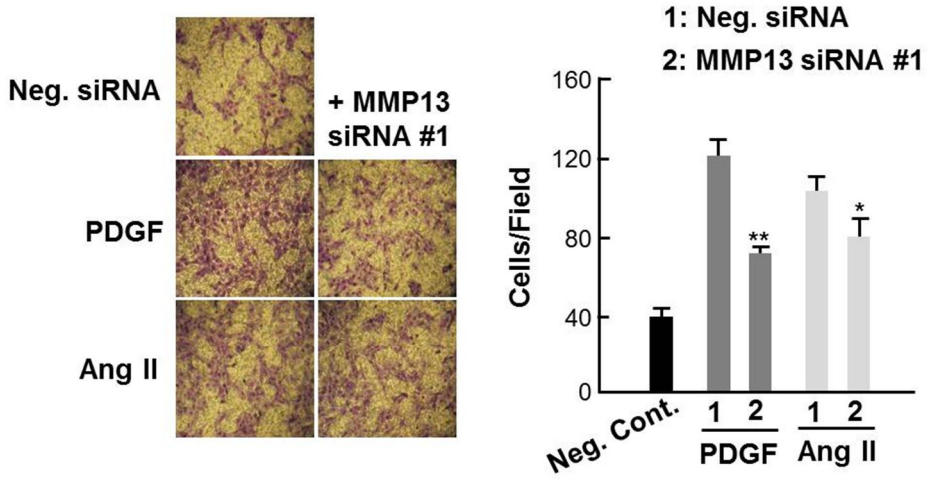

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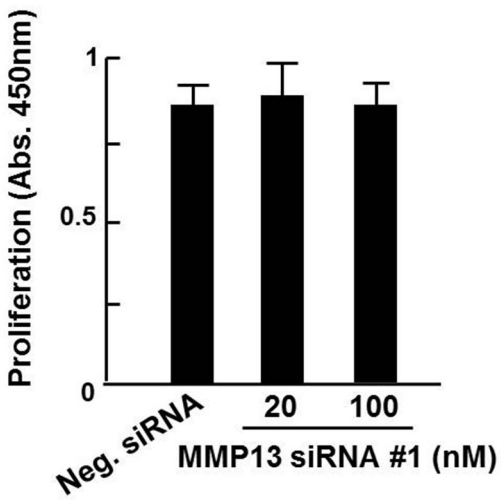

Figure 17. MMP13 silencing in RAoSMCs. RAoSMCs were transfected with MMP13 siRNA or control siRNA using silent Fect. After 24 h transfection, cells and media were harvested for Western blotting, RNA extraction, zymographic analysis, or Boyden chamber assays. (A) MMP13 silencing by three different MMP13 siRNAs in RAoSMCs. The same amounts of lysates were analyzed by Western blotting using an antibody against MMP13. Anti-actin blot was used as a loading control. (B) mRNA levels of MMP13 were quantified by real-time PCR after treatment control or MMP13 siRNAs. (C) Extracellular activity of MMP13 after treatment control or MMP13 siRNAs was analyzed using zymography. (D) RAoSMCs migration after treatment control or MMP13 siRNAs was analyzed using Boyden chamber assays. Columns, average of triple determinations; bars, SD. *P < 0.05 or**P < 0.01 as compared with Neg. siRNA-treated control.

3.3.3. MMP13 is downregulated by a known RAoSMCs migration inhibitor, cordycepin

Cordycepin is a type of nucleoside analog isolated from Cordyceps militaris, which inhibits VSMC migration via down regulation of MMP2 and MMP9 [38, 102]. Therefore, we used cordycepin to confirm whether MMP13 plays a role in VSMC migration. Similar to previous reports, treatment with PDGF increased intracellular MMP2 and MMP9 expression levels as well as their secreted levels in the medium, which were significantly decreased by cordycepin in a dose-dependent manner. Interestingly, we observed a similar trend in MMP13 expression levels. The secreted level of MMP13 was ~2-fold higher than that of the untreated control but no significant difference was observed in cell lysates. Treatment with cordycepin decreased MMP13 expression by up to 50% in both the media and cell lysates (Fig. 18A and B). Furthermore, the increased mRNA levels of MMP13 by PDGF treatment were inhibited by cordycepin (Fig. 18C). In addition to the result that silencing MMP13 significantly decreased the migration of RAoSMCs (Fig. 17D), these results indicate that MMP13 might be one of mediators in VSMCs migration.

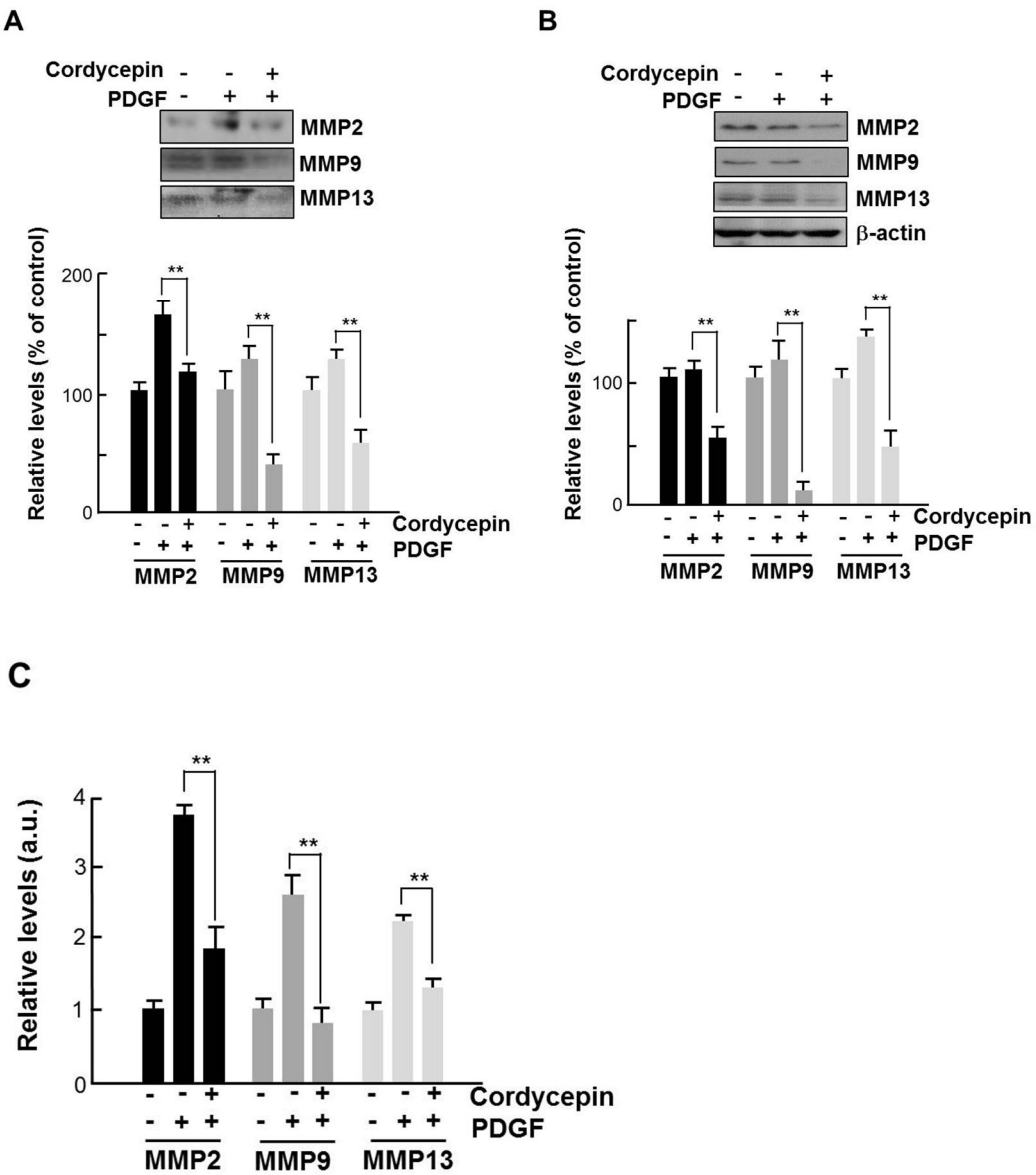

Figure 18. Altered MMP13 expression by cordycepin. (A) Altered MMPs expression by cordycepin was evaluated in media and (B) cell lysates of 5 ng PDGF-treated RAoSMCs using Western blot. (C) The effects of cordycepin on mRNA levels of MMP13 were quantified by real-time PCR in PDGF-induced RAoSMCs. Columns, average of triple determinations; bars, SD. **P < 0.01 as compared with PDGF-treated control.

3.3.4. Akt and ERK are potential mediators of MMP13 expression

The signal transduction pathways that modulate the activity of MMP transcription factors are highly diverse. Many reports have demonstrated that mitogen-activated protein kinase (MAPK) signal transduction pathways, including p38, ERK, and JNK, are well known mediators that stimulate or inhibit MMP expression depending on the cell types [88, 103]. To understand the mechanism by which stimuli regulate MMP13 expression in VSMCs, we analyzed the effects of signal mediator inhibitors on MMP13 expression. We found that an Akt or ERK-specific inhibitors, but not PI3K inhibitor, significantly decreased MMP13 expression levels (Fig. 19). These results indicate that Akt and ERK are likely upstream mediators that regulate MMP13 expression and functions in cell migration of VSMCs.

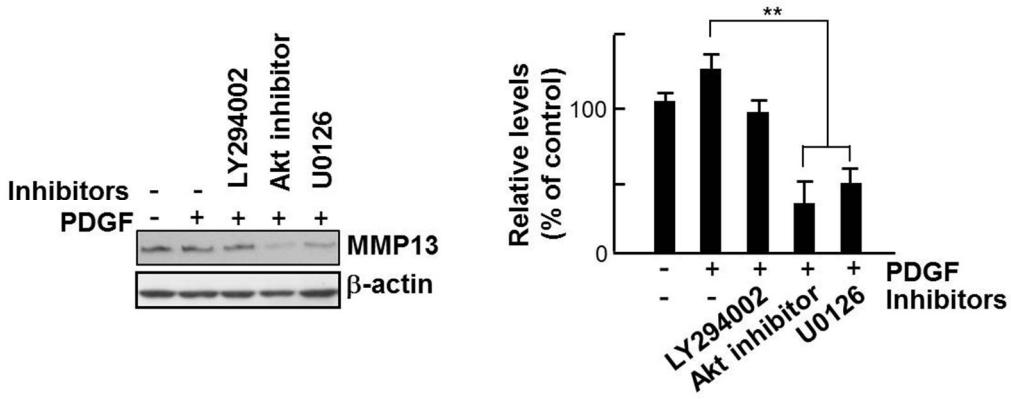

Figure 19. Effects of inhibitors on MMP13 expression. The resultant MMP13 expressions in the absence or presence of 5 μM LY294002, 2 μM Akt inhibitor, or 5 μM U0126 were revealed using Western blot. Columns, average of triple determinations; bars, SD. **P < 0.01 as compared with non-treated control.

Ⅳ. Discussion

In the present study, we show that the extracts from *D. morbifera* lead to a significant decrease of RAoSMCs proliferation and migration and provide evidences that the extracts regulate the transcriptional levels of MMP2 and MMP9 in RAoSMCs. We further show that the extracts from *D. morbifera* regulation of MMP2 and MMP9 expression is likely mediated by Akt and ERK signal pathways. Even though the disparity of the effects of the extracts from *D. morbifera* on proliferation, 2-D, and 3-D migration occurred, we could observe the obvious significant effects of the extracts on the proliferation and migration of RAoSMCs. Also we show that the extracts from *D. morbifera* led to a significant decrease of vascular intimal hyperplasia in balloon-injured rat carotid arteries. Several lead compounds, such as lycopene (from tomatoes), resveratrol (from grapes and peanuts), sulforaphane (from asparagus), allicin (from garlic), EGCG (from green tea) are in preclinical or clinical trials for cancer chemoprevention [104]. Because of their safety, low toxicity, antioxidant properties, and general acceptance as dietary supplements, fruits, vegetables, and other dietary elements are being investigated for the prevention of cancer. Also many epidemiological studies have shown that diets rich in natural phenolic compounds reduce the risk of cardiovascular diseases. In addition, many reports demonstrate that *D.morbifera* have anti-plasmodial, anti-atherogenic, anti-diabetic, anti-cancer effects [42-44]. The *D.morbifera* stem extract facilitates cadmium excretion from the blood, kidney tissues of cadmium-exposed rats, inhibits cadmium-induced oxidative stress in the brain tissues by increasing antioxidant levels after cadmium exposure. The ethanol extract of *D.morbifera* markedly inhibited the growth of human leukemia-U937 cells by decreasing cell proliferation and inducing apoptosis through the caspase-dependent

pathway [105, 106]. Further since its investigation in 2009 by Moon et al, many reports demonstrate about *D.morbifera* that *D.morbifera* essential oil has significant lipid-lowering effects. All five compounds that oleifoliosides A (1) and B (2), dendropanoxide (3), beta-amyrin (4) and alpha-amyrin (5) have been isolated from the stem parts of *D.morbifera* , of that , compounds 2 and 3 showed notable growth inhibitory activity against chloroquine-sensitive strains of Plasmodium falciparum on SK-OV-3 cancer cell lines. Dendropanoxide in the streptozotocin-induced diabetic rats showed significant hypoglycemic activity for 14 days. The antidiabetic effects of the dendropanoxide was more effective than that observed with glibenclamide, a known antidiabetic drug. Oleifolioside A, a new triterpenoid compound isolated from *D.morbifera* induced apoptosis in HeLa cells involving nuclear translocation of mitochondrial apoptogenic factors AIF and EndoG. Also attenuated LPS-stimulated inducible nitric oxide synthase (iNOS) and cycloxigenase-2 (COX-2) expression through the downregulation of NF-kB and MAPK activities in RAW 264.7 macrophages. Oleifolioside B, a cycloartane-type triterpene glycoside isolated from *D.morbifera* induced autophagy functions as a death mechanism in A549cells, exposure to oleifolioside B promotes apoptosis [41, 42, 44, 107, 108]. Dendropanoxide induces autophagy through ERK1/2 activation in MG-63 human osteosarcoma cells and autophagy inhibited cell proliferation and induced apoptosis [43]. However, the effects of *D.morbifera* on cardiovascular diseases have not yet been reported.

In our finding, by orally administrated 90mg/kg/d, extract from *D.morbifera* could histopathology considerably improve that much change in neointima : decrease the intimal area, the ratio from intima to media and increase the lumen area. The extract from *D.morbifera* inhibited PCNA and MMP2 expression in intima. In this study, we found that pre-treated groups (during 2 weeks, treated *D.morbifera* before balloon injury) had a markedly lower expression as compared to the control group (data not shown). We further observed that mRNA

expression level of PCNA and MMP2 significantly decreased. Thus, the extract from *D.morbifera* on intima formation, at least in part, have inhibitory effects on the proliferation and migration of VSMCs.

Conclusively, our results provide strong evidence that reducing neointima formation in balloon-injured rat carotid arteries by the extracts of *D.morbifera* may protect against early atherogenesis, particularly VSMC proliferation and migration. Further, investigation will be needed to identify the detailed mechanisms of inhibitory effects of extracts from *D. morbifera* in VSMCs that promising possibility for the treatment to atherogenic incidences.

In this study, the effects of the extracts of *D. morbifera* on hypoxia-reoxygenation injured cardiomyocytes (CMC) were investigated. The condition of cardiomyocytes with hypoxia (30min) and reoxgenation (1hr) decreased the cell number, but the treatment of *D. morbifera* inhibited CMC death induced apoptosis. These results suggested that the progress of apoptosis in CMC are regulated by *D. morbifera* after hypoxia/ reoxygenation. *D. morbifera* treated CMC reduced ROS generation and intracellular calcium concentration ([Ca(2+)]i) compared with positive control (the condition of hypoxia-reoxygenation). Also DM attenuated abnormal changes of RyR2 and SERCA2a genes in hypoxic cardiomyocytes by western blot. These results suggest that DM ameliorates ROS generation and Ca(2+)i homeostasis as preventing dysregulation of calcium regulatory proteins in the heart, thereby producing the cardioprotective effect and reduction in hypoxic cardiomyocytes damage.

In order to screen the natural products having activities for cardiovascular diseases, 31 more extracts besides *D. morbifera* were tested. Then, each extracts were tested whether they have anti-proliferative and anti-migratory activities on RAoSMCs. As these results, indicated that the activity of *P. asiatica* is specific on anti-proliferation and those of *P. densiflora* is specific on anti-migration in RAoSMCs. Although many reports demonstrate that *P. asiatica*

have hypocholesterolaemic effects in mice and cytotoxic, anti-viral and immunomodulatory effects [81, 83, 109], the role of *P. asiatica* in migratory or proliferative VSMCs has been unclear. Also the earlier studies found that *P. densiflora* induces apoptosis through ROS generation and activation of caspases in YD-8 human oral cancer cells and inhibits invasion and migration of SK-Hep-1 hepatocellular carcinoma cells [110, 111]. But the role of *P. densiflora* in migratory or proliferative VSMCs has been unclear. Therefore, further studies will be needed to reveal the mechanisms underlying the effects of the extracts on the regulation of RAoSMCs.

In this study, we showed that MMP13 silencing led to a prominent decrease in PDGF- and Ang II-induced RAoSMCs migration, which is a major event in the early course of atherosclerosis [112], and provided evidence that MMP13 participated in this progression. Although many reports demonstrate that MMP13 is involved in migration and invasion on various tumor and non-tumor cell types, such as esophageal squamous cell car-cinoma, cutaneous squamous cell carcinoma, and endothelial cells [113-115], the migratory role of MMP13 in VSMCs has been unclear. Our finding that Ang II treatment resulted in a important increase of MMP13 expression in RAoSMCs is similar to the previous report in which Ang II induced production of MMP3 and MMP13 through the AT1 receptor in osteoblasts [116]. The diversity of MMP13 expression and role among these studies may reflect the various characteristics of the experimental systems including stimuli, cell types, and signaling pathways that participate in cellular migration. For all that, we found that PDGF treatment resulted in a prominent increase of transcriptional and translational MMP13 levels, indicating that MMP13 may be a mediator of RAoSMCs migration besides MMP2 and MMP9. This result is supported by the actual fact that inhibition of MMP13 activity led to a important decrease of RAoSMCs migration and co-suppression of MMP13 activity with MMP2 or

MMP9 showed synergistic effects on the decrease of RAoSMCs migration. MMP13 Silencing by a specific small interfering RNA (siRNA) remarkably decreased expression of active form of MMP13, which is followed by the migration of decrease on PDGF-or Ang II-treated RAoSMCs. Interestingly, the study observed synergistic inhibitory effects in migration by treatment with MMP2 and 13 or MMP9 and 13 inhibitors compared to the treatment of single. Moreover, found that cordycepin, a known inhibitor of migration in VSMC, caused significant downregulation of MMP2, 9, and 13 expression in PDGF-treated RAoSMCs. To understand the mechanism by which stimulus regulate MMP13 expression in VSMCs, analyzed the inhibitory effects of signal mediator on MMP13 expression and found that an Akt or ERK-specific inhibitors significantly decreased MMP13 expression levels, but not PI3K inhibitor. Our results that inhibition or silencing of MMP13 success-enough decreased cell migration of PDGF- or Ang II-treated RAoSMCs strongly suggests that MMP13 expression levels are positively correlated with RAoSMCs migration rates. Indeed, these results are corresponded with the previous report that B16F1 melanoma grafts displayed reduced tumor growth and significantly decreased metastasis and angiogenesis in a MMP13−/− mouse model compared towildtype mice [117]. Our observation provide strong evidence that reducing MMP13 expression and function may preserve against early atherogenesis, particularly VSMC migration. Additionally, because MT1-MMP (MMP14) is a known activator of both MMP2 and 13 [118], more research will be needed to disclose the relationship of MT1-MMP or MMP2 and MMP13 in atherogenesis. In addition, further studies aimed at identifying the precise signal transduction pathways and transcription factors that mediate MMP13 expression may provide a molecular understanding of the roles of MMP13 in atherosclerosis.

Ⅴ. References

1. Mendis S, Lindholm LH, Mancia G, Whitworth J, Alderman M, Lim S, Heagerty T: **World Health Organization (WHO) and International Society of Hypertension (ISH) risk prediction charts: assessment of cardiovascular risk for prevention and control of cardiovascular disease in low and middle-income countries**. *J Hypertens* 2007, **25**(8):1578-1582.
2. http://www.who.int/cardiovascular_diseases/en
3. Owens GK, Kumar MS, Wamhoff BR: **Molecular regulation of vascular smooth muscle cell differentiation in development and disease**. *Physiol Rev* 2004, **84**(3):767-801.
4. Ross R: **Cell biology of atherosclerosis**. *Annu Rev Physiol* 1995, **57**:791-804.
5. Wang Z, Ahmad A, Li Y, Kong D, Azmi AS, Banerjee S, Sarkar FH: **Emerging roles of PDGF-D signaling pathway in tumor development and progression**. *Biochim Biophys Acta* 2010, **1806**(1):122-130.
6. Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A: **Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype**. *J Biol Chem* 2009, **284**(6):3728-3738.
7. Ross R: **The pathogenesis of atherosclerosis: a perspective for the 1990s**. *Nature* 1993, **362**(6423):801-809.
8. Lee HM, Jeon BH, Won KJ, Lee CK, Park TK, Choi WS, Bae YM, Kim HS, Lee SK, Park SH *et al*: **Gene transfer of redox factor-1 inhibits neointimal formation: involvement of platelet-derived growth factor-beta receptor signaling via the inhibition of the reactive oxygen species-mediated Syk pathway**. *Circ Res* 2009, **104**(2):219-227, 215p following 227.
9. Song H, Li Y, Lee J, Schwartz AL, Bu G: **Low-density lipoprotein receptor-related protein 1 promotes cancer cell migration and invasion by inducing the expression of matrix metalloproteinases 2 and 9**. *Cancer Res* 2009, **69**(3):879-886.
10. Newby AC: **Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates**. *Cardiovasc Res* 2006, **69**(3):614-624.
11. Dey NB, Lincoln TM: **Possible involvement of Cyclic-GMP-dependent protein kinase on matrix metalloproteinase-2 expression in rat aortic smooth muscle cells**. *Mol Cell Biochem* 2012, **368**(1-2):27-35.
12. Chien YC, Sheu MJ, Wu CH, Lin WH, Chen YY, Cheng PL, Cheng HC: **A Chinese herbal formula "Gan-Lu-Yin" suppresses vascular smooth muscle cell migration by inhibiting matrix metalloproteinase-2/9 through the PI3K/AKT and ERK signaling pathways**. *BMC Complement Altern Med* 2012, **12**:137.
13. Karki R, Jeon ER, Kim DW: **Nelumbo nucifera leaf extract inhibits neointimal hyperplasia through modulation of smooth muscle cell proliferation and**

migration. *Nutrition* 2013, **29**(1):268-275.
14. Gurjar MV, Sharma RV, Bhalla RC: **eNOS gene transfer inhibits smooth muscle cell migration and MMP-2 and MMP-9 activity**. *Arterioscler Thromb Vasc Biol* 1999, **19**(12):2871-2877.
15. Williams DO, Holubkov R, Yeh W, Bourassa MG, Al-Bassam M, Block PC, Coady P, Cohen H, Cowley M, Dorros G *et al*: **Percutaneous coronary intervention in the current era compared with 1985-1986: the National Heart, Lung, and Blood Institute Registries**. *Circulation* 2000, **102**(24):2945-2951.
16. Bavry AA, Kumbhani DJ, Helton TJ, Bhatt DL: **Risk of thrombosis with the use of sirolimus-eluting stents for percutaneous coronary intervention (from registry and clinical trial data)**. *Am J Cardiol* 2005, **95**(12):1469-1472.
17. Nilsen DW, Melberg T, Larsen AI, Barvik S, Bonarjee V: **Late complications following the deployment of drug eluting stents**. *Int J Cardiol* 2006, **109**(3):398-401.
18. Muto A, Fitzgerald TN, Pimiento JM, Maloney SP, Teso D, Paszkowiak JJ, Westvik TS, Kudo FA, Nishibe T, Dardik A: **Smooth muscle cell signal transduction: implications of vascular biology for vascular surgeons**. *J Vasc Surg* 2007, **45 Suppl A**:A15-24.
19. Lee MY, Griendling KK: **Redox signaling, vascular function, and hypertension**. *Antioxid Redox Signal* 2008, **10**(6):1045-1059.
20. Szocs K, Lassegue B, Sorescu D, Hilenski LL, Valppu L, Couse TL, Wilcox JN, Quinn MT, Lambeth JD, Griendling KK: **Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury**. *Arterioscler Thromb Vasc Biol* 2002, **22**(1):21-27.
21. Griendling KK, FitzGerald GA: **Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS**. *Circulation* 2003, **108**(16):1912-1916.
22. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gomez-Gracia E, Ruiz-Gutierrez V, Fiol M, Lapetra J *et al*: **Primary prevention of cardiovascular disease with a Mediterranean diet**. *N Engl J Med* 2013, **368**(14):1279-1290.
23. de Lorgeril M, Renaud S, Mamelle N, Salen P, Martin JL, Monjaud I, Guidollet J, Touboul P, Delaye J: **Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease**. *Lancet* 1994, **343**(8911):1454-1459.
24. Stein EA, Raal FJ: **New therapies for reducing low-density lipoprotein cholesterol**. *Endocrinol Metab Clin North Am* 2014, **43**(4):1007-1033.
25. Rai AK, Debetto P, Sala FD: **Molecular regulation of cholesterol metabolism: HDL-based intervention through drugs and diet**. *Indian J Exp Biol* 2013, **51**(11):885-894.
26. Dauchet L, Amouyel P, Hercberg S, Dallongeville J: **Fruit and vegetable consumption and risk of coronary heart disease: a meta-analysis of cohort studies**. *J Nutr* 2006, **136**(10):2588-2593.

27. He FJ, Nowson CA, MacGregor GA: **Fruit and vegetable consumption and stroke: meta-analysis of cohort studies**. *Lancet* 2006, **367**(9507):320-326.
28. Renaud S, de Lorgeril M: **Wine, alcohol, platelets, and the French paradox for coronary heart disease**. *Lancet* 1992, **339**(8808):1523-1526.
29. Kuriyama S, Shimazu T, Ohmori K, Kikuchi N, Nakaya N, Nishino Y, Tsubono Y, Tsuji I: **Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study**. *JAMA* 2006, **296**(10):1255-1265.
30. Schini-Kerth VB, Etienne-Selloum N, Chataigneau T, Auger C: **Vascular protection by natural product-derived polyphenols: in vitro and in vivo evidence**. *Planta Med* 2011, **77**(11):1161-1167.
31. Orozco-Sevilla V, Naftalovich R, Hoffmann T, London D, Czernizer E, Yang C, Dardik A, Dardik H: **Epigallocatechin-3-gallate is a potent phytochemical inhibitor of intimal hyperplasia in the wire-injured carotid artery**. *J Vasc Surg* 2013, **58**(5):1360-1365.
32. Piao CS, Kim DS, Ha KC, Kim HR, Chae HJ, Chae SW: **The Protective Effect of Epigallocatechin-3 Gallate on Ischemia/Reperfusion Injury in Isolated Rat Hearts: An ex vivo Approach**. *Korean J Physiol Pharmacol* 2011, **15**(5):259-266.
33. Zhang C, Lin G, Wan W, Li X, Zeng B, Yang B, Huang C: **Resveratrol, a polyphenol phytoalexin, protects cardiomyocytes against anoxia/reoxygenation injury via the TLR4/NF-kappaB signaling pathway**. *Int J Mol Med* 2012, **29**(4):557-563.
34. Yue R, Hu H, Yiu KH, Luo T, Zhou Z, Xu L, Zhang S, Li K, Yu Z: **Lycopene protects against hypoxia/reoxygenation-induced apoptosis by preventing mitochondrial dysfunction in primary neonatal mouse cardiomyocytes**. *PLoS One* 2012, **7**(11):e50778.
35. Chan JY, Tsui HT, Chung IY, Chan RY, Kwan YW, Chan SW: **Allicin protects rat cardiomyoblasts (H9c2 cells) from hydrogen peroxide-induced oxidative injury through inhibiting the generation of intracellular reactive oxygen species**. *Int J Food Sci Nutr* 2014, **65**(7):868-873.
36. Xia Q, Wang X, Xu DJ, Chen XH, Chen FH: **Inhibition of platelet aggregation by curdione from Curcuma wenyujin essential Oil**. *Thromb Res* 2012, **130**(3):409-414.
37. Cheng H, Liu W, Ai X: **[Protective effect of curcumin on myocardial ischemia reperfusion injury in rats]**. *Zhong Yao Cai* 2005, **28**(10):920-922.
38. Chang W, Lim S, Song H, Song BW, Kim HJ, Cha MJ, Sung JM, Kim TW, Hwang KC: **Cordycepin inhibits vascular smooth muscle cell proliferation**. *Eur J Pharmacol* 2008, **597**(1-3):64-69.
39. Jung SM, Park SS, Kim WJ, Moon SK: **Ras/ERK1 pathway regulation of p27KIP1-mediated G1-phase cell-cycle arrest in cordycepin-induced inhibition of the proliferation of vascular smooth muscle cells**. *Eur J Pharmacol* 2012, **681**(1-3):15-22.

40. Park ES, Kang DH, Yang MK, Kang JC, Jang YC, Park JS, Kim SK, Shin HS: **Cordycepin, 3'-deoxyadenosine, prevents rat hearts from ischemia/reperfusion injury via activation of Akt/GSK-3beta/p70S6K signaling pathway and HO-1 expression**. *Cardiovasc Toxicol* 2014, **14**(1):1-9.
41. Yu HY, Kim KS, Lee YC, Moon HI, Lee JH: **Oleifolioside A, a New Active Compound, Attenuates LPS-Stimulated iNOS and COX-2 Expression through the Downregulation of NF-kappaB and MAPK Activities in RAW 264.7 Macrophages**. *Evid Based Complement Alternat Med* 2012, **2012**:637512.
42. Chung IM, Kim MY, Park SD, Park WH, Moon HI: **In vitro evaluation of the antiplasmodial activity of Dendropanax morbifera against chloroquine-sensitive strains of Plasmodium falciparum**. *Phytother Res* 2009, **23**(11):1634-1637.
43. Lee JW, Kim KS, An HK, Kim CH, Moon HI, Lee YC: **Dendropanoxide induces autophagy through ERK1/2 activation in MG-63 human osteosarcoma cells and autophagy inhibition enhances dendropanoxide-induced apoptosis**. *PLoS One* 2013, **8**(12):e83611.
44. Jin CY, Yu HY, Park C, Han MH, Hong SH, Kim KS, Lee YC, Chang YC, Cheong J, Moon SK *et al*: **Oleifolioside B-mediated autophagy promotes apoptosis in A549 human non-small cell lung cancer cells**. *Int J Oncol* 2013, **43**(6):1943-1950.
45. Lim L, Yun JJ, Jeong JE, Wi AJ, Song H: **Inhibitory Effects of Nano-Extract from Dendropanax morbifera on Proliferation and Migration of Vascular Smooth Muscle Cells**. *J Nanosci Nanotechnol* 2015, **15**(1):116-119.
46. Li Q, Kobayashi M, Wakayama Y, Inagaki H, Katsumata M, Hirata Y, Hirata K, Shimizu T, Kawada T, Park BJ *et al*: **Effect of phytoncide from trees on human natural killer cell function**. *Int J Immunopathol Pharmacol* 2009, **22**(4):951-959.
47. Abe T, Hisama M, Tanimoto S, Shibayama H, Mihara Y, Nomura M: **Antioxidant effects and antimicrobial activites of phytoncide**. *Biocontrol Sci* 2008, **13**(1):23-27.
48. Seo EJ, Lee SY, Kang SS, Jung YS: **Zizyphus jujuba and its active component jujuboside B inhibit platelet aggregation**. *Phytother Res* 2013, **27**(6):829-834.
49. Suzuki Y, Kondo K, Ikeda Y, Umemura K: **Antithrombotic effect of geniposide and genipin in the mouse thrombosis model**. *Planta Med* 2001, **67**(9):807-810.
50. Ku SK, Kim TH, Lee S, Kim SM, Bae JS: **Antithrombotic and profibrinolytic activities of isorhamnetin-3-O-galactoside and hyperoside**. *Food Chem Toxicol* 2013, **53**:197-204.
51. Cheng J, Kondo K, Suzuki Y, Ikeda Y, Meng X, Umemura K: **Inhibitory effects of total flavones of Hippophae Rhamnoides L on thrombosis in mouse femoral artery and in vitro platelet aggregation**. *Life Sci* 2003, **72**(20):2263-2271.
52. Hsu JH, Wu YC, Liu IM, Cheng JT: **Release of acetylcholine to raise insulin secretion in Wistar rats by oleanolic acid, one of the active principles contained in Cornus officinalis**. *Neurosci Lett* 2006, **404**(1-2):112-116.
53. Koo YK, Kim JM, Koo JY, Kang SS, Bae K, Kim YS, Chung JH, Yun-Choi HS: **Platelet anti-aggregatory and blood anti-coagulant effects of compounds**

isolated from Paeonia lactiflora and Paeonia suffruticosa. *Pharmazie* 2010, 65(8):624-628.

54. Louis XL, Murphy R, Thandapilly SJ, Yu L, Netticadan T: Garlic extracts prevent oxidative stress, hypertrophy and apoptosis in cardiomyocytes: a role for nitric oxide and hydrogen sulfide. *BMC Complement Altern Med* 2012, 12:140.
55. Suetsuna K, Maekawa K, Chen JR: Antihypertensive effects of Undaria pinnatifida (wakame) peptide on blood pressure in spontaneously hypertensive rats. *J Nutr Biochem* 2004, 15(5):267-272.
56. Gu J, Wang JJ, Yan J, Cui CF, Wu WH, Li L, Wang ZS, Yu M, Gao N, Liu L *et al*: Effects of lignans extracted from Eucommia ulmoides and aldose reductase inhibitor epalrestat on hypertensive vascular remodeling. *J Ethnopharmacol* 2011, 133(1):6-13.
57. Ryu SY, Oh KS, Kim YS, Lee BH: Antihypertensive, vasorelaxant and inotropic effects of an ethanolic extract of the roots of Saururus chinensis. *J Ethnopharmacol* 2008, 118(2):284-289.
58. Higashino S, Sasaki Y, Giddings JC, Hyodo K, Sakata SF, Matsuda K, Horikawa Y, Yamamoto J: Crocetin, a carotenoid from Gardenia jasminoides Ellis, protects against hypertension and cerebral thrombogenesis in stroke-prone spontaneously hypertensive rats. *Phytother Res* 2014, 28(9):1315-1319.
59. Rodrigo R, Gonzalez J, Paoletto F: The role of oxidative stress in the pathophysiology of hypertension. *Hypertens Res* 2011, 34(4):431-440.
60. Zhu F, Huang B, Hu CY, Jiang QY, Lu ZG, Lu M, Wang MH, Gong M, Qiao CP, Chen W *et al*: Effects of total flavonoids of Hippophae rhamnoides L. on intracellular free calcium in cultured vascular smooth muscle cells of spontaneously hypertensive rats and Wistar-Kyoto rats. *Chin J Integr Med* 2005, 11(4):287-292.
61. Liu F, Wei Y, Yang XZ, Li FG, Hu J, Cheng RF: [Hypotensive effects of safflower yellow in spontaneously hypertensive rats and influence on plasma renin activity and angiotensin II level]. *Yao Xue Xue Bao* 1992, 27(10):785-787.
62. Choi DH, Lee YJ, Kim JS, Kang DG, Lee HS: Cynanchum wilfordii ameliorates hypertension and endothelial dysfunction in rats fed with high fat/cholesterol diets. *Immunopharmacol Immunotoxicol* 2012, 34(1):4-11.
63. Karki R, Jeon ER, Kim DW: Magnoliae Cortex inhibits intimal thickening of carotid artery through modulation of proliferation and migration of vascular smooth muscle cells. *Food Chem Toxicol* 2012, 50(3-4):634-640.
64. Karki R, Ho OM, Kim DW: Magnolol attenuates neointima formation by inducing cell cycle arrest via inhibition of ERK1/2 and NF-kappaB activation in vascular smooth muscle cells. *Biochim Biophys Acta* 2013, 1830(3):2619-2628.
65. Karki R, Kim SB, Kim DW: Magnolol inhibits migration of vascular smooth muscle cells via cytoskeletal remodeling pathway to attenuate neointima formation. *Exp Cell Res* 2013, 319(20):3238-3250.

66. Kim S, Kim CK, Lee KS, Kim JH, Hwang H, Jeoung D, Choe J, Won MH, Lee H, Ha KS *et al*: **Aqueous extract of unripe Rubus coreanus fruit attenuates atherosclerosis by improving blood lipid profile and inhibiting NF-kappaB activation via phase II gene expression**. *J Ethnopharmacol* 2013, **146**(2):515-524.
67. Wu Q, Wang W, Li S, Nagarkatti P, Nagarkatti M, Windust A, Wang XL, Tang D, Cui T: **American ginseng inhibits vascular smooth muscle cell proliferation via suppressing Jak/Stat pathway**. *J Ethnopharmacol* 2012, **144**(3):782-785.
68. Son JE, Hwang MK, Lee E, Seo SG, Kim JE, Jung SK, Kim JR, Ahn GH, Lee KW, Lee HJ: **Persimmon peel extract attenuates PDGF-BB-induced human aortic smooth muscle cell migration and invasion through inhibition of c-Src activity**. *Food Chem* 2013, **141**(4):3309-3316.
69. Shen M, Wu RX, Zhao L, Li J, Guo HT, Fan R, Cui Y, Wang YM, Yue SQ, Pei JM: **Resveratrol attenuates ischemia/reperfusion injury in neonatal cardiomyocytes and its underlying mechanism**. *PLoS One* 2012, **7**(12):e51223.
70. Jiang WL, Zhang SM, Tang XX, Liu HZ: **Protective roles of cornuside in acute myocardial ischemia and reperfusion injury in rats**. *Phytomedicine* 2011, **18**(4):266-271.
71. Han SY, Li HX, Ma X, Zhang K, Ma ZZ, Tu PF: **Protective effects of purified safflower extract on myocardial ischemia in vivo and in vitro**. *Phytomedicine* 2009, **16**(8):694-702.
72. Duan JL, Wang JW, Guan Y, Yin Y, Wei G, Cui J, Zhou D, Zhu YR, Quan W, Xi MM *et al*: **Safflor yellow A protects neonatal rat cardiomyocytes against anoxia/reoxygenation injury in vitro**. *Acta Pharmacol Sin* 2013, **34**(4):487-495.
73. Townsend PA, Scarabelli TM, Pasini E, Gitti G, Menegazzi M, Suzuki H, Knight RA, Latchman DS, Stephanou A: **Epigallocatechin-3-gallate inhibits STAT-1 activation and protects cardiac myocytes from ischemia/reperfusion-induced apoptosis**. *FASEB J* 2004, **18**(13):1621-1623.
74. Ji G, Yao X, Zang Z, Huang Z: **[Antiarrhythmic effect of Oenanthe javanica (Bl.) DC. injection]**. *Zhongguo Zhong Yao Za Zhi* 1990, **15**(7):429-431, 448.
75. Lim L, Jang YS, Yun JJ, Song H: **Phytoncide, Nanochemicals from Chamaecyparis obtusa, Inhibits Proliferation and Migration of Vascular Smooth Muscle Cells**. *J Nanosci Nanotechnol* 2015, **15**(1):112-115.
76. Kwon JH, Kim JH, Choi SE, Park KH, Lee MW: **Inhibitory effects of phenolic compounds from needles of Pinus densiflora on nitric oxide and PGE2 production**. *Arch Pharm Res* 2010, **33**(12):2011-2016.
77. Jung MJ, Choi JH, Chung HY, Jung JH, Choi JS: **A new C-methylated flavonoid glycoside from Pinus densiflora**. *Fitoterapia* 2001, **72**(8):943-945.
78. Choi EM: **Antinociceptive and antiinflammatory activities of pine (Pinus densiflora) pollen extract**. *Phytother Res* 2007, **21**(5):471-475.
79. Ince I, Yesil-Celiktas O, Karabay-Yavasoglu NU, Elgin G: **Effects of Pinus brutia bark extract and Pycnogenol in a rat model of carrageenan induced inflammation**. *Phytomedicine* 2009, **16**(12):1101-1104.

80. Kwak CS, Moon SC, Lee MS: **Antioxidant, antimutagenic, and antitumor effects of pine needles (Pinus densiflora)**. *Nutr Cancer* 2006, **56**(2):162-171.
81. Chung MJ, Park KW, Kim KH, Kim CT, Baek JP, Bang KH, Choi YM, Lee SJ: **Asian plantain (Plantago asiatica) essential oils suppress 3-hydroxy-3-methyl-glutaryl-co-enzyme A reductase expression in vitro and in vivo and show hypocholesterolaemic properties in mice**. *Br J Nutr* 2008, **99**(1):67-75.
82. Foresti R, Hoque M, Monti D, Green CJ, Motterlini R: **Differential activation of heme oxygenase-1 by chalcones and rosolic acid in endothelial cells**. *J Pharmacol Exp Ther* 2005, **312**(2):686-693.
83. Chiang LC, Chiang W, Chang MY, Lin CC: **In vitro cytotoxic, antiviral and immunomodulatory effects of Plantago major and Plantago asiatica**. *Am J Chin Med* 2003, **31**(2):225-234.
84. Yang SW, Lim L, Ju S, Choi DH, Song H: **Effects of matrix metalloproteinase 13 on vascular smooth muscle cells migration via Akt-ERK dependent pathway**. *Tissue Cell* 2015, **47**(1):115-121.
85. No JH, Jo H, Kim SH, Park IA, Kang D, Lee CH, Han SS, Kim JW, Park NH, Kang SB *et al*: **Expression of MMP-2, MMP-9, and urokinase-type plasminogen activator in cervical intraepithelial neoplasia**. *Ann N Y Acad Sci* 2009, **1171**:100-104.
86. Galis ZS, Sukhova GK, Lark MW, Libby P: **Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques**. *J Clin Invest* 1994, **94**(6):2493-2503.
87. Westermarck J, Kahari VM: **Regulation of matrix metalloproteinase expression in tumor invasion**. *FASEB J* 1999, **13**(8):781-792.
88. Johansson N, Ala-aho R, Uitto V, Grenman R, Fusenig NE, Lopez-Otin C, Kahari VM: **Expression of collagenase-3 (MMP-13) and collagenase-1 (MMP-1) by transformed keratinocytes is dependent on the activity of p38 mitogen-activated protein kinase**. *J Cell Sci* 2000, **113 Pt 2**:227-235.
89. Buja LM, Entman ML: **Modes of myocardial cell injury and cell death in ischemic heart disease**. *Circulation* 1998, **98**(14):1355-1357.
90. Hearse DJ: **Reperfusion of the ischemic myocardium**. *J Mol Cell Cardiol* 1977, **9**(8):605-616.
91. Thompson JA, Hess ML: **The oxygen free radical system: a fundamental mechanism in the production of myocardial necrosis**. *Prog Cardiovasc Dis* 1986, **28**(6):449-462.
92. Griendling KK, FitzGerald GA: **Oxidative stress and cardiovascular injury: Part II: animal and human studies**. *Circulation* 2003, **108**(17):2034-2040.
93. Barry WH, Bridge JH: **Intracellular calcium homeostasis in cardiac myocytes**. *Circulation* 1993, **87**(6):1806-1815.
94. Pierce GN, Russell JC: **Regulation of intracellular Ca2+ in the heart during diabetes**. *Cardiovasc Res* 1997, **34**(1):41-47.
95. Choi KM, Zhong Y, Hoit BD, Grupp IL, Hahn H, Dilly KW, Guatimosim S, Lederer WJ, Matlib MA: **Defective intracellular Ca(2+) signaling contributes to**

cardiomyopathy in Type 1 diabetic rats. *Am J Physiol Heart Circ Physiol* 2002, 283(4):H1398-1408.

96. Boudina S, Abel ED: **Diabetic cardiomyopathy revisited**. *Circulation* 2007, 115(25):3213-3223.
97. Trost SU, Belke DD, Bluhm WF, Meyer M, Swanson E, Dillmann WH: **Overexpression of the sarcoplasmic reticulum Ca(2+)-ATPase improves myocardial contractility in diabetic cardiomyopathy**. *Diabetes* 2002, 51(4):1166-1171.
98. Bidasee KR, Dincer UD, Besch HR, Jr.: **Ryanodine receptor dysfunction in hearts of streptozotocin-induced diabetic rats**. *Mol Pharmacol* 2001, 60(6):1356-1364.
99. Shao CH, Rozanski GJ, Patel KP, Bidasee KR: **Dyssynchronous (non-uniform) Ca2+ release in myocytes from streptozotocin-induced diabetic rats**. *J Mol Cell Cardiol* 2007, 42(1):234-246.
100. Kain V, Kumar S, Sitasawad SL: **Azelnidipine prevents cardiac dysfunction in streptozotocin-diabetic rats by reducing intracellular calcium accumulation, oxidative stress and apoptosis**. *Cardiovasc Diabetol* 2011, 10:97.
101. Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, Bartlett RK, Lowe JS, O'Donnell SE, Aykin-Burns N *et al*: **A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation**. *Cell* 2008, 133(3):462-474.
102. Tuli HS, Sharma AK, Sandhu SS, Kashyap D: **Cordycepin: a bioactive metabolite with therapeutic potential**. *Life Sci* 2013, 93(23):863-869.
103. Nakai K, Kawato T, Morita T, Iinuma T, Kamio N, Zhao N, Maeno M: **Angiotensin II induces the production of MMP-3 and MMP-13 through the MAPK signaling pathways via the AT(1) receptor in osteoblasts**. *Biochimie* 2013, 95(4):922-933.
104. Gullett NP, Ruhul Amin AR, Bayraktar S, Pezzuto JM, Shin DM, Khuri FR, Aggarwal BB, Surh YJ, Kucuk O: **Cancer prevention with natural compounds**. *Semin Oncol* 2010, 37(3):258-281.
105. Kim W, Kim DW, Yoo DY, Jung HY, Nam SM, Kim JW, Hong SM, Kim DW, Choi JH, Moon SM *et al*: **Dendropanax morbifera Leveille extract facilitates cadmium excretion and prevents oxidative damage in the hippocampus by increasing antioxidant levels in cadmium-exposed rats**. *BMC Complement Altern Med* 2014, 14:428.
106. Lee JW, Park C, Han MH, Hong SH, Lee TK, Lee SH, Kim GY, Choi YH: **Induction of human leukemia U937 cell apoptosis by an ethanol extract of Dendropanax morbifera Lev. through the caspase-dependent pathway**. *Oncol Rep* 2013, 30(3):1231-1238.
107. Moon HI: **Antidiabetic effects of dendropanoxide from leaves of Dendropanax morbifera Leveille in normal and streptozotocin-induced diabetic rats**. *Hum Exp Toxicol* 2011, 30(8):870-875.
108. Yu HY, Jin CY, Kim KS, Lee YC, Park SH, Kim GY, Kim WJ, Moon HI, Choi YH,

Lee JH: **Oleifolioside A mediates caspase-independent human cervical carcinoma HeLa cell apoptosis involving nuclear relocation of mitochondrial apoptogenic factors AIF and EndoG**. *J Agric Food Chem* 2012, **60**(21):5400-5406.

109. Yamada H, Nagai T, Takemoto N, Endoh H, Kiyohara H, Kawamura H, Otsuka Y: **Plantagoside, a novel alpha-mannosidase inhibitor isolated from the seeds of Plantago asiatica, suppresses immune response**. *Biochem Biophys Res Commun* 1989, **165**(3):1292-1298.
110. Jo JR, Park JS, Park YK, Chae YZ, Lee GH, Park GY, Jang BC: **Pinus densiflora leaf essential oil induces apoptosis via ROS generation and activation of caspases in YD-8 human oral cancer cells**. *Int J Oncol* 2012, **40**(4):1238-1245.
111. Lee SJ, Lee KW, Hur HJ, Chun JY, Kim SY, Lee HJ: **Phenolic phytochemicals derived from red pine (Pinus densiflora) inhibit the invasion and migration of SK-Hep-1 human hepatocellular carcinoma cells**. *Ann N Y Acad Sci* 2007, **1095**:536-544.
112. Doran AC, Meller N, McNamara CA: **Role of smooth muscle cells in the initiation and early progression of atherosclerosis**. *Arterioscler Thromb Vasc Biol* 2008, **28**(5):812-819.
113. Lopez-Rivera E, Lizarbe TR, Martinez-Moreno M, Lopez-Novoa JM, Rodriguez-Barbero A, Rodrigo J, Fernandez AP, Alvarez-Barrientos A, Lamas S, Zaragoza C: **Matrix metalloproteinase 13 mediates nitric oxide activation of endothelial cell migration**. *Proc Natl Acad Sci U S A* 2005, **102**(10):3685-3690.
114. Xu N, Zhang L, Meisgen F, Harada M, Heilborn J, Homey B, Grander D, Stahle M, Sonkoly E, Pivarcsi A: **MicroRNA-125b down-regulates matrix metallopeptidase 13 and inhibits cutaneous squamous cell carcinoma cell proliferation, migration, and invasion**. *J Biol Chem* 2012, **287**(35):29899-29908.
115. Ye Q, Yan Z, Liao X, Li Y, Yang J, Sun J, Kawano T, Wang X, Cao Z, Wang Z *et al*: **MUC1 induces metastasis in esophageal squamous cell carcinoma by upregulating matrix metalloproteinase 13**. *Lab Invest* 2011, **91**(5):778-787.
116. Nakamura M, Yao M, Sano F, Sakata R, Tatenuma T, Makiyama K, Nakaigawa N, Kubota Y: **[A case of metastatic renal cell carcinoma associated with Birt-Hogg-Dube syndrome treated with molecular-targeting agents]**. *Hinyokika Kiyo* 2013, **59**(8):503-506.
117. Zigrino P, Kuhn I, Bauerle T, Zamek J, Fox JW, Neumann S, Licht A, Schorpp-Kistner M, Angel P, Mauch C: **Stromal expression of MMP-13 is required for melanoma invasion and metastasis**. *J Invest Dermatol* 2009, **129**(11):2686-2693.
118. Knauper V, Bailey L, Worley JR, Soloway P, Patterson ML, Murphy G: **Cellular activation of proMMP-13 by MT1-MMP depends on the C-terminal domain of MMP-13**. *FEBS Lett* 2002, **532**(1-2):127-130.

Ⅵ. Acknowledgements

First of all, I am so grateful to my God. I thank to all your kindles help for doctorial course.

I would like to express my gratitude to all those who gave me the possibility to complete this thesis.

I especially want to express the deepest appreciation to my committee, Professor Heesang Song, for his guidance of the manuscript and deeply appreciate, leading all of my matters and studies for upgrade of my life.

I want to thank them for all their help, support, interest, valuable hints, and generous supports. especially I thank to my colleagues Sujin Ju, Juyong Jo, Seulki Park for their efforts.

On a personal note I would like to thank my family and all my friends. In particular, I would like to express my sincere thanks to my husband, three daughters, mother in law, and my parents who constantly provided emotional support and took care of me in many aspects.

December, 2015

Leejin Lim