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Inhibitory activity of *Dendropanax morbiferum* extracts against liver fibrosis

조선대학교 대학원

의 학 과

김 서 진

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황칠나무 추출물의 간섬유화 억제 활성

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Abbreviations

α-SMA, α-smooth muscle actin

AML-2/DX100, doxorubicin-resistant acute myelogenous leukemia cell line

ALT, alanine aminotransferase

AST, aspartate aminotransferase

ATP, adenosine triphosphate

BSA, bovine serum albumin

CTGF, connective tissue growth factor

CTL, cytolytic T lymphocyte

DCFH-DA, 2',7'-dichloro fluorescindiacetate

DM, *Dendropanax morbifera*

DMB, *Dendropanax morbifera* butyl alcohol extract

DMEA, *Dendropanax morbifera* ethylacetate extract

DMEM, Dulbecco's modified Eagle's media

DMH, *Dendropanax morbifera* hexane extract

DMM, *Dendropanax morbifera* methanol extract

DMW, *Dendropanax morbifera* aqueous extract

dNTPs, deoxyribonucleoside triphosphates

DPPH, 2,2-diphenyl-1-picrylhydrazyl

ECM, extracellular matrix

EMT, epithelial-mesenchymal transition

FBS, fetal bovine serum

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Hs27, human dermal fibroblast cell

HSCs, hepatic stellate cells

NAD, nicotinamide adenine dinucleotide

PBS, phosphate-buffered saline

PCR, polymerase chain reaction

PDGF, platelet-derived growth factor

PMSF, phenylmethylsulfonyl fluoride

ROS, reactive oxygen species

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

TGF-ß1, transforming growth factor ß1

국 문 초 록

황칠나무 추출물의 간섬유화 억제 활성

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배경: 간섬유화는 조절되지 않으면 간경화까지 일으키는 간암의 주된 원인이지만 효과적인 치료가 아직 없는 실정이다. 산화적 손상과 같은 만성 간 손상에서 간성상세포(hepatic satellate cells, HSCs)가 콜라겐과 같은 세포외 기질을 생산함으로써 간 섬유화의 병인에 중요한 역할을 한다. 본 연구에서는 강력한 항산화 효과를 가지고 있는 황칠나무 추출물의 항섬유화 효과를 활성화된 HSCs와 사염화탄소(CCl_4)로 유도한 흰쥐 간 섬유화 모델에서 확인하고자 하였다.

방법: 황칠 잎을 전통적인 액체-액체 추출방법으로 추출하였다. HSCs는 transforming growth factor-beta(TGF-ß)로 활성화시켰으며 alpha-smooth muscle actin(α-SMA), connective tissue growth factor(CTGF) 및 fibronectin-extra domain A(FN-EDA)의 유전자발현을 RT-PCR 및 Western blot 분석법 또는 면역조직화학 염색법으로 측정하였다. 황칠추출물의 항산화 효과는 시험관에서 2,2-diphenyl-1-picrylhydrazyl (DPPH) 방법과 세포에서 2',7'-dichloro fluorescin diacetate(DCFH-DA) 방법으로 측정하였다. Scratch 상처 방법으로 HSCs 및 진피 섬유아세포(Hs27)의 이동을 측정하였다. 30% ethanol로 90℃ 에서 추출한 추출물(DMEE)의 CCl_4로 유도한 흰쥐 간 섬유화 모델에서 간보호 및 항섬유화 효과를 silymarin (SM)과 비교하였다. 총 21마리의 수컷 Sprague-Dawley 흰쥐를 4개의 군으로 분류하였다. (1)용매군 (carboxymethylcellulose, CMC 및 올리브 오일), (2)CMC 및 CCl_4 군 , (3)CCl_4투여 30분전 DMEE 경구 투여군 (100 mg/kg체중), (4)CCl_4투여 30분전 SM 경구 투여군(100 mg/kg체중). 일주일에 두 번 6주간 투여후 간독성을 측정하기 위해 복부정맥에서

혈액을 뽑아 혈청을 분리하였고, 섬유화를 확인하기 위해 간 조직을 절제하여 Hematoxylin 및 Eosin(H&E) 염색과 콜라겐을 관찰하기 위하여 sirius red 염색을 하였다.

결과: 모든 황칠추출물이 HSCs에서 TGF-β1으로 유도한 α-SMA mRNA 발현을 현저하게 억제하였으며, 추출물중에서 헥산추출물(DMH)가 가장 높은 억제효과를 보였다. DMH가 TGF-β1로 유도된 α-SMA, CTGF, FN-EDA의 유전자 발현을 농도 의존적으로 억제하는 것을 RT-PCR 및 Western blot 분석으로 확인하였고, α-SMA의 발현은 면역조직화학 염색법으로 다시 확인하였다. DMEE의 안전성, 추출수율, 항산화 효능을 고려하여 동물실험의 후보추출물 원료로 사용하였다. DMEE도 HSCs와 Hs27에서 섬유화의 지표유전자인 α-SMA과 CTGF mRNAs의 발현을 농도 의존적으로 억제하였다. DMEE는 HSCs와 Hs27의 증식과 이동을 농도 의존적으로 억제하였다. SD 흰쥐에서 DMEE 현탁액(100 mg/kg)을 6주간 주당 2회 CCl_4 복강내 투여전 30분에 경구로 전처치한 경우 CCl_4에 의한 체중 감소를 억제하였으며, 마지막 CCl_4투여후 다음 날 혈청의 AST(aspartate aminotransferase) 와 ALT(alanine aminotransferase)의 증가의 감소가 양성대조군인 실리마린보다 우수하였다. 간조직의 염색에서 DMEE와 실리마린 투여군은 CCl_4 단독 투여군과 비교시 간세포의 소포화 변화가 적었고 혈관주변으로 방사되는 콜라겐 섬유 격막이 약하고 불연속적으로 관찰되었다.

결론: 황칠나무추출물은 시험관에서 HSCs의 활성화와 이동을 억제할 뿐만 아니라 CCl_4로 유도한 흰쥐 간 섬유화 모델에서 간독성과 콜라겐 축적을 억제하였다. 그러므로 강력한 항산화 효능을 가진 황칠나무 추출물은 간섬유화를 예방하거나 치료에 도움이 될 수 있을 것으로 생각된다.

I. Introduction

Liver fibrosis represents a major health problem accounting for the death of nearly one million people worldwide (Sanchez-Valle *et al*. 2012). The condition can be induced by chronic liver injury through natural and experimental causes including virus infection, alcohol abuse, radiation, cholestasis, oxidative stress, toxic chemicals (such as CCl4 and nitrosamine), drugs (such as stilbestrol and methyldopa), and a choline-deficient diet (Gebhardt 2002). During chronic liver injury, activated hepatic stellate cells (HSCs, hepatic lipocytes, fat-storing cells, Ito cells, perisinusoidal cells) proliferate, resulting in excessive deposition of extracellular matrix (ECM) proteins, such as collagen and fibronectin, attributing to liver fibrosis (Duval *et al*. 2014). When such a fibrosis is not controlled, it can progress into end-stage cirrhosis and/or hepatocellular carcinoma. A recent finding revealed that liver fibrosis and cirrhosis could be reversible if their causes could be weakened or removed (Hansen and Christensen 2015). Nevertheless, there is no effective antifibrotic regimen to date. It has been reported that reactive oxygen species (ROS) are involved in liver damage and liver fibrosis (Luangmonkong *et al*. 2018). Since plants with various biological activities are relatively safe and cost-effective, medicinal plant-derived antioxidants can shed light on the prevention and treatment of liver fibrosis and cirrhosis.

Dendropanax morbifera Leveille (DM)is an evergreen plant mainly distributed in the southwest regions and islands of South Korea (Park *et al*. 2004). It is well known as one of the elixir plants or “herb of eternal youth” as designated by the King Qin Shi Huang of the state of Qin in China (260-210 BC). Its scientific name is composed of dendro (tree as Greek) + panax (pan, all + axos, cure or drug as Greek), morbi (disease as Latin) + ferus (moving as Latin) and Leveille (French surname), meaning a panacea (cure-all) treating many kinds of diseases. This name is appropriate based on the many efficacies described in the historical literature and peer-reviewed scientific papers as an antioxidant, as well as applications to cancer (colon cancer, osteosarcoma, hepatoma, non-small

cell lung cancer, cervical cancer and leukemia), diabetes mellitus, inflammatory diseases, atherosclerosis, Parkinson's disease, and gout (Kim *et al*. 2018b; Kim *et al*. 2015; Moon 2011; Park *et al*. 2018; Song *et al*. 2018).

This study investigated preventive and/or treatment applications of DM extracts with potent antioxidant and antifibrotic effects both *in vitro,* using HSCs, and *in vivo,* using a CCl_4-induced liver fibrosis rat model.

II. Materials and Methods

1. Preparation of DM extracts

DM leaves were dried and then extracted using 10-volume methanol 3 times at room temperature. The methanol extracts were concentrated using a rotary vacuum evaporator and filtered through a Whatman No.1 filter, then resuspended in water. Finally, the extracts were further successively partitioned with n-hexane and ethylacetate through liquid-liquid extraction method. DM ethanol extracts were obtained using 30% ethanol at 25℃, 60℃, and 90℃, 50% ethanol at 25℃ and 70% at 25℃.

2. Cell culture

The fibroblast Hs27 cells and HSCs were cultured in Dulbecco's modified Eagle's media (DMEM), supplemented with 10% heat inactivated fetal bovine serum (FBS), and 100 μg/ml antibiotics in a 5% CO_2 incubator humidified at 37℃. The cells were maintained as a monolayer culture and subcultured at confluence.

3. RT-PCR assay

Total cellular RNA was extracted using MagExtractor® for the MFX-2100 (Toyobo, Osaka, Japan) auto-nucleic acid purification system. RNA (1 μg) was reverse transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen, USA) and an oligo (dT) primer for 1 hr at 37℃. Real-Time PCR was performed with the Light Cycler 2.0 thermocycler (Roche, Mannheim, Germany) using the Taqman Master Mix (Roche). Each reaction mixture contained diluted cDNA, 10 pmol of each primer and probe, and master mix containing buffer, dNTPs, $MgCl_2$, and Tag polymerase. Primers used for real-time PCR are listed in Table1.

4. Western blot analysis

Protein concentration was determined using Bio-Rad protein assay kit on the basis of the Bradford method (Schleicher and Wieland 1978). Standards or samples (4 μL) were added into 1 ml of 5-fold diluted dye reagent and absorbance was measured at 595 nm. Sample protein concentration was determined from standard curve prepared from bovine serum albumin (BSA).

The HSCs were washed with phosphate-buffered saline (PBS) and lysed in 50 mMTris-Cl (pH 7.4), 250 mM NaCl, 0.5% Triton X100, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). Protein expression was assessed in the cell lysates by SDS-PAGE and Western blotting. Membranes were incubated with primary rabbit polyclonal antibodies specific for α-SMA (Abcam, USA), CTGF (Abcam, USA), FN-EDA (Santa Cruz, USA) and GAPDH (Santa Cruz, USA). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 2h at room temperature against each host IgG for primary antibodies. The membranes were then stained using the detection reagent of the ECL detection kit (Amersham, USA).

5. Immunohistochemistry

Immunohistochemistry was performed using a diaminobenzidine (DAB)-based staining technique as described previously elsewhere. Briefly, HSCs on a slide were fixed with 3.7% formaldehyde for 30min and then permeabilized with PBS-0.2% Triton X-100 for 10 min, and incubated with 3% fatty acid-free bovine serum albumin (BSA) (Sigma) in PBS-0.1% Triton X-100 (PBST) for 1h to block non-specific staining. Cells were incubated with a primary antibody against α-SMA (1:100, Abcam) and then with a biotinylated secondary antibody. α-SMA expression was visualized by incubating cells in DAB and counterstaining with Mayer's hematoxylin, washed, dehydrated in alcohol, cleared in xylene and mounted with Histomount (National Diagnostics, USA) and covered with a coverslip.

6. Determination of cell-free antioxidant activity using DPPH assay

Free radical-scavenging activity was measured using DPPH radicals (Kedare and Singh 2011). The assay mixture was composed with 3.98 ml methanol, 20 μl of each extract, and 1 ml DPPH (0.15 mM in methanol). The mixture was incubated at room temperature for 30 min, and then absorbance was measured at 517 nm using a spectrophotometer (UV-1800, Shimadzu, Japan). The experiment was performed in triplicate. The IC_{50} value meaning 50% inhibition of DPPH radicals was calculated from a graph of radical-scavenging activity versus extract concentrations.

7. Determination of intracellular antioxidant activity using DCFH-DA assay

DCFH-DA fluorometric assay is on the basis of the principal that a non-fluorescent probe DCFH-DA diffused into cells is hydrolyzed by cytoplasmic esterase to non-fluorescent 2,7′ -dichlorofluorescin(DCFH),which is then oxidized by reactive oxygen species (ROS) to fluorescent 2,7′ -dichlorofluorescein (DCF) (Aranda *et al*. 2013). In this study, doxorubicin-resistant AML-2 cells supersensitive to hydrogen peroxide were used for enhanced sensitivity (Kim *et al*. 2001). AML-2/DX100 cells (1×10^5 cells/ml) were suspended in PBS at 37° C and placed in a humidified incubator containing 5% CO_2. Following a 30 min incubation, the cells were treated with 1μM DCFH-DA with 4 mM hydrogen peroxide for 2h. The levels of DCF were measured using a spectrofluorometer (PerkinElmer, USA) on a n excitation wave length at485 nm and emission at 530 nm.

8. Scratch-wound assay

A scratch-wound assay was performed to measure cell migration (Cory 2011). Hs27 and HSCs were seeded at 1×10^5 cells/well in 6-well plates and were allowed to grow to confluence. A thin wound was made by scratching the surface with a

20-μl pipette tip, and the wounded cells were washed twice with phosphate-buffered saline (PBS). Cells were then incubated with medium containing DM extracts. The migration speed into the cell free zone was calculated as a mean distance of three (short, medium, and long) selected cell free zone.

9. Administration schedule in CCl_4-induced rat liver fibrosis model

This animal study was approved by the Chosun University Institutional Animal Care and Use Committee. The animals were obtained from the Dammul Science (Daejeon, Korea), and were housed in the SPF facility with a 12h light and 12h dark schedule and fed autoclaved chow and water. The animals were weighed twice a week for determination of bodyweight change and readjustment of the CCl_4 and DM extract treatment amounts.

Carboxylmethyl cellulose (CMC) was used as a suspension vehicle (4 ml/kg of body weight) for oral administration. CCl_4 was diluted in olive oil (30%) and administered through intraperitoneal injection at 2 ml/kg of body weight twice a week. The rats were pretreated with CMC, DM ethanol extract, and Silymarin for 30 min before CCl_4 administration. A total of 21 pathogen-free male Sprague-Dawley rats (body weight: 200-250 g) were employed in the study, which were divided into 4 groups: (1) vehicle (CMC and olive oil) group (n = 5), (2) CMC and CCl_4 group (n = 6), (3) oral DM ethanol extract pretreatment (100 mg/kg/body weight) before CCl_4group (n = 5) and (4) oral Silymarin pretreatment (100 mg/kg/body weight) before CCl_4 group (n = 5). After 6 weeks, all rats were sacrificed to prepare serum and liver samples for hepatotoxicity and histopathological examination. Blood samples were obtained from the abdominal vein. The levels of ALT and AST were measured by the GPT and GOT kit (Asan, Korea) according to the manufacturer's instructions.

For histological study, the tissues were fixed with formalin overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and Sirius Red for collagen I and III staining using the staining kit (ScyTek,

USA) as per manufacturer's protocols.

10. Statistical analysis

Each experiment was done at least three times, and data are expressed as the means ± SE. Statistical significance was determined by paired Student's *t* test. p-values < 0.05 were considered statistically significant.

III. Results and Discussion

In chronic liver injury, such as oxidative stress, HSCs play a pivotal role in the pathogenesis of liver fibrosis by producing ECM proteins, such as collagen. In this study, DM extracts with strong antioxidant effects were investigated for antifibrotic effects in activated HSCs *in vitro* and a CCl_4-induced rat liver fibrosis model.

DM leaves were extracted and fractionated using conventional liquid-liquid extraction methods. Extraction yields and antioxidant effects of the DM extracts are summarized in Table 2. As shown in Table 2, extraction yields of methanol, hexane, ethylacetate, and water are 14.2%, 1.9%, 0.9%, and 24.1%, respectively. Extraction yields of 30%, 50%, and 70% ethanol at room temperature were 7.6%, 21.0%, and 19.7%, respectively. Increasing the extraction temperature from 25℃ to 60℃ and 90℃ in the case of 30% ethanol enhanced the yields by 68.4% and 309.2%, respectively. These results indicate that extraction yield with 30% ethanol at 90℃ is approximately equivalent to that with 70% ethanol at room temperature.

Since liver fibrosis involves excessive oxidative stress, DM extracts could exhibit an antifibrotic effect through antioxidant activity. Antioxidant effects were determined using the DPPH assay in a cell-free system and the DCFH-DA assay in cells, which accounts for the difference in membrane penetration of the active antioxidant components according to their lipid solubility. The DPPH assay revealed that DM extracts obtained with methanol, hexane, ethylacetate, and water showed less fewer antioxidant effects than those of the DM ethanol extracts. Ethanol is believed to be the best extraction solvent for preserving antioxidant activities, which are relatively dependent upon the ethanol concentrations. These results are consistent with other reports that an 80% ethanol DM extract showed higher radical scavenging activity, and higher total phenol and flavonoid content (Nakamura *et al.* 2016). On the other hand, the DCFH-DA assay revealed that

30% ethanol, ethylacetate, and hexane DM extracts showed higher radical scavenging activity than extracts obtained with 50% and 70% ethanol extracts, methanol, and water acts. These results indicate that intracellular antioxidant activity is negatively correlated with ethanol concentration. The difference between DPPH assay is a cell-free assay, while the DCFH-DA assay is a cell-based assay. The intracellular antioxidant activity in the DCFH-DA assay is dependent on the lipid solubility of the major antioxidant components extracted with each solvent. Therefore, the antioxidant effect of phytochemicals should be evaluated in cell-free systems, as well as in cells.

The antioxidant effects of medicinal plants are generally related to the polyphenols and/or flavonoids (Seo *et al*. 2016). Recent studies demonstrate that chlorogenic acid has antioxidant properties, reducing the relative risk of cardiovascular diseases and providing hepatoprotective effects against acetaminophen toxicity (Kim *et al*. 2018a). Additionally, the flavonoidrutin has also been shown to be associated with antioxidative effects (Pan *et al*. 2014). A recent study has shown that a DM butanol extract exhibits a relatively higher concentration of phenolic-flavonoids, including chlorogenic acid (1.95%), rutin (0.64%), and others such asvitexin, quercetin, caffeic acid, tricin, ferulic acid, kaempferol, and luteolin (Park *et al*. 2018). Additional research has shown the extraction efficiencies of chlorogenic acid and rutinare positively correlated with the concentrations of ethanol, where an 80% ethanolic DM extract contained 4.7% chlorogenic acid and 3.3% rutin (Seo *et al*. 2016). This result is consistent with another report showing that an 80% ethanol DM extract would be the ideal extracting solution to maximize antioxidant activity (Nakamura *et al*. 2016). In this study, DM leave extracts obtained with 30% ethanol at 90℃ contained 0.7% chlorogenic acid and 0.9% rutin, where 95% ethanol at the room temperature contained 1.7% chlorogenic acid and 0.3% rutin. These results indicated that major phenolic-flavonoids components, such as chlorogenic acid and rutin, may represent possess antioxidant effects. However, the extraction efficiency of the polyphenols and/or flavonoids could vary with components and extraction solvents.

Differentiation of quiescent HSCs into active myofibroblasts, which secrete excessive amounts of ECM proteins, is critical in liver fibrosis (Kumar *et al*. 2016). Transforming growth factor-beta1 (TGF-β1) is considered to be the main profibrotic cytokine responsible for the activation of HSCs (Xie *et al*. 2015).It is known that α-SMA is not only a useful marker for the detection of liver fibrosis but also an important marker of HSC activation (Hirabaru *et al*. 2014; Parajuli *et al*. 2015). In this study, HSCs were activated by TGF-β1 (1 ng/ml) and the expression of α-SMA mRNA was measured using an RT-PCR assay. As shown in Fig 1, TGF-β1 (1 ng/ml) increased α-SMA mRNA significantly in HSCs, while all DM extracts of 50 μg/ml inhibited TGF-β1-induced α-SMA mRNA expression to the level observed in cells that received no TGF-β1 treatment. Of the DM extracts, the hexane extract (DMH) showed the strongest effect. Concentration dependence was tested with DMH at 2, 10, and 50 μg/ml. As shown in Fig. 2, RT-PCR and Western blot analyses showed that DMH inhibited TGF-β1-induced α-SMA gene expression in a concentration-dependent manner. Silymarin (SM) has a strong antioxidant effect and was used as a positive control, also inhibiting TGF-β1-induced α-SMA gene expression in HSCs. Immunohistochemical staining also showed that DMH inhibited the level of α-SMA protein as much as the SM control (Fig. 3). These results indicate the antifibrotic effect of DMH could be due to reduced HSCs activation and the downregulated expression of α-SMA.

CTGF, a key determinant of fibrosis, is upregulated in various fibrotic diseases including liver fibrosis (Blom *et al*. 2002). As a profibrotic marker, CTGF acts as a downstream effector of TGF-β1 and is a target for antifibrotic therapies inhibiting the profibrotic effects of TGF-β1 (Blom *et al*. 2002). As shown in Fig 4,RT-PCR and Western blot analyses revealed that DMH significantly inhibited TGF-β1-induced CTGF gene expression to below the level observed in cells that received no TGF-β1 treatment in a concentration-dependent fashion. CTGF is also a fibrogenic master switch for the epithelial-mesenchymal transition (ECM), which is associated with liver fibrosis (Gressner and Gressner 2008). Therefore, it remains to be determined whether DM extracts might inhibit ECM by increasing epithelial markers, such as

E-cadren,and decreasing mesenchymal markers, such as vimentin.

Fibrosis is characterized by excessive accumulation of collagen and other ECM components that are actively involved in fibrosis (Duval *et al*. 2014). HSC maintenance is dependent on the ECM, such as the fibronectin splice variant containing the extra domain A (FN-EDA) (Wight and Potter-Perigo 2011). Fig 5 shows that TGF-β1 (1 ng/ml) significantly increased FN-EDA mRNA and protein levels in HSCs and DMH inhibited TGF-β1-induced FN-EDA gene expression in a concentration-dependent manner. Conversely, SM inhibited TGF-β1-induced FN-EDA mRNA expression but not TGF-β1-induced FN-EDA production. These results demonstrate the effect of DMH on FN-EDA expression is different from that of SM. DMEE was also tested for inhibition of α-SMA and CTGF mRNA expression. As shown in Fig 6, DMEE also inhibited α-SMA and CTGF mRNA expression in the absence of TGF-β1, even though its effect was weaker than DMH.

The cost-effectiveness ratio is of significant importance for commercialization. The DM water extract has the highest extraction yield but the lowest effectiveness. Although DM hexane and ethylacetate extracts showed the highest antioxidant activities, they should be re-evaluated for their toxicity and low extraction yield. Among them, the DM extract obtained with 30% ethanol at 90℃ showed the highest extraction yield on the basis of cellular antioxidant activity. These results indicate that the DM extract with 30% ethanol at 90℃ (DMEE) is a good candidate DM extract material for antioxidant activities, considering the extraction yield reflecting the cost. Therefore, DMEE was used for further study with the expectation of future commercialization.

The influence of DMEE on the migration of HSCs and human dermal fibroblast Hs27 cells was tested using a wound-healing scratch assay. Two repeated experiments showed that DMEE decreased the closure rate of a wound area of HSCs and Hs27 cells in a concentration-dependent manner (Fig. 7 and Fig. 8). The types of compounds involved in delaying wound healing by inhibiting migration and/or proliferation of fibroblasts remains to be determined what. Chlorogenic acid was reported to inhibit the proliferation of HSCs and the

generation of ECM components, such as collagen, resulting in antifibrosis(Shi *et al*. 2015).Rutin is also a potential inhibitor of the mTOR pathway,which activates HSCs, and may contribute to liver fibrosis (Thiyagarajan *et al*. 2017). Therefore, it could be suggested that chlorogenic acid and rutin, major phenolic-flavonoid components of DM extracts, might be involved in inhibition of fibroblast migration.

Chronic CCl_4 administration has been commonly used to induce liver fibrosis in experimental systems through its metabolic transformation into trichloromethyl free radicals resulting in serious liver injury (Marques *et al*. 2012).

After termination of the experiment, the vehicle and CCl_4 group showed body weight (BW)increment by 9.5% and 5.5%,respectively, as compared with BW prior to treatment. These results demonstrate that CCl_4 inhibits normal BW gain according to age. Groups pretreated orally with DMEE and SM prior to CCl_4 administration showed BW increment by 8.5% and 6.5%,indicating that DMEE is more effective than SM for BW improvement.

It is well known that ALT and AST are intracellular enzymes which can leak out into plasma when hepatocytes are damaged by inflammation and toxicants (Kamei *et al*. 1986). At the end of the study, serum levels of ALT and AST were determined. CCl_4 administration increased the levels of serum ALT and AST by 2.7-fold and 2.4-fold as compared with that of the vehicle control. Pretreatment with DMEE (100 mg/kg of BW) decreased the serum levels of ALT and AST by 54% and 45%, respectively, compared to those of CCl_4 administration. Pretreatment with SM (100 mg/kg of BW) decreased the serum levels of ALT and AST by 60% and 72.3%, respectively, compared to those of CCl_4 administration (Fig. 9). Although not considered statistically significant, these results indicate that DMEE is more effective in protecting against CCl_4-induced hepatotoxicity than SM.

In staining experiments, Sirius Red shows collagen as red in color. The vehicle control group showed that fibrotic components, such as collagen, were limited to the central veins (Fig 10, CMC + oil), but CCl_4 administration

resulted in severe vacuolar changes of the hepatocytes and complete fibrotic septae interconnecting from central to central veins and from central to portal veins (Fig.10, CMC+CCl_4). DMEE and SM (100 mg/kg of body weight) treated groups showed less vacuolar change and little deposition of collagen fibers in the liver compared with the livers of CCl_4 group (Fig.10). Chlorogenic acid protects against CCl_4-induced liver fibrosis through the suppression of oxidative stress in HSCs and the liver through the inhibition of the TLR4/MyD88/NF-κB signaling pathway(Shi *et al*. 2013; Shi *et al*. 2016). The antifibrotic effect of rutin is not only associated with antioxidant and anti-inflammatory effects but also the downregulation of NF-κB and TGF-β/Smad signaling through inhibition of ERK activation and/or enhancement of Nrf2, HO-1, and AMPK activity in a cholestaticrat liver model produced by bile duct ligation (Pan *et al*. 2014).

Taken together, these results suggest that the antifibrotic effect of DM extracts may be related to antioxidant activity due to the presence of polyphenols and flavonoids, such as chlorogenic acid and rutin. In conclusion, DM extracts can effectively inhibit liver fibrosis not only by inhibiting HSC activation and migration *in vitro* but also by preventing hepatotoxicity and hepatic collagen accumulation *in vivo*. Thus, DM extracts with strong antioxidant effects may shed light on the prevention and treatment of liver fibrosis.

Table 1. Primers and conditions for real-time PCR

Gene	Primer	Sequence
α-SMA	Sense	5'-TAGCTGAGCGTGGCTATT-3'
	Antisense	5'-CCCATCAGGCACTCGTA-3'
CTGF	Sense	5'-CGAGGAGTGGGTGTGTGA-3'
	Antisense	5'-CCCACAGGTCTTGGAAC-3'
FN-EDA	Sense	5'-TCCAAGCGGAGAGAGT-3'
	Antisense	5'-GTGGGTGTGACCTGAG-3'
GAPDH	Sense	5'-CGAGATCCCTCCAAAATCAA-3'
	Antisense	5'-TGTGGTCATGAGTCCTTCCA-3'

α-SMA, alpha-smooth muscle actin; CTGF, connective tissue growth factor; FN-EDA, fibronectin extradomain A; GAPDH, glycealdehyde phosphate dehydrogenase

Table 2. Extraction yields and antioxidant effect of DM extracts according to extraction solvents and temperature

		MeOH	Hexane	EA	D.W	30% EtOH			50% EtOH	70% EtOH
						90℃	60℃	25℃		
Extraction yield (%)		14.2	1.9	0.9	24.1	23.5	12.8	7.6	21.0	19.7
Antioxidant effects	DPPH radical-scavenging activity (IC_{50}, μg/ ml)	148	290	143	122	75.4	69.8	63.1	69.1	58
	DCFH-DA radical-scavenging activity (IC_{50} μg/ml)	27.0	13.4	10.9	31.2	15.9	10.3	10.0	19.5	24

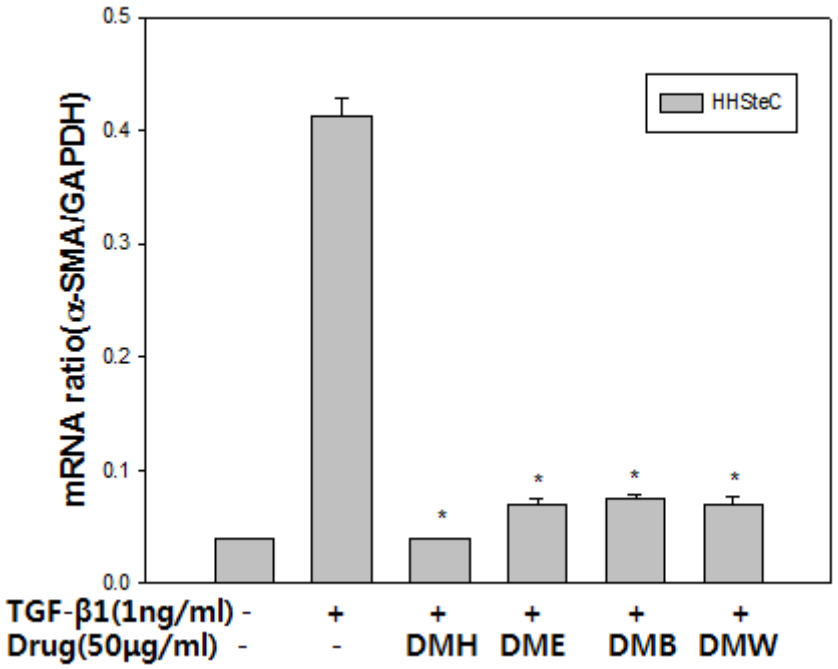

Fig. 1. Effect of DM extracts on TGF-β1-induced α-SMA mRNA expression. * P < 0.05.

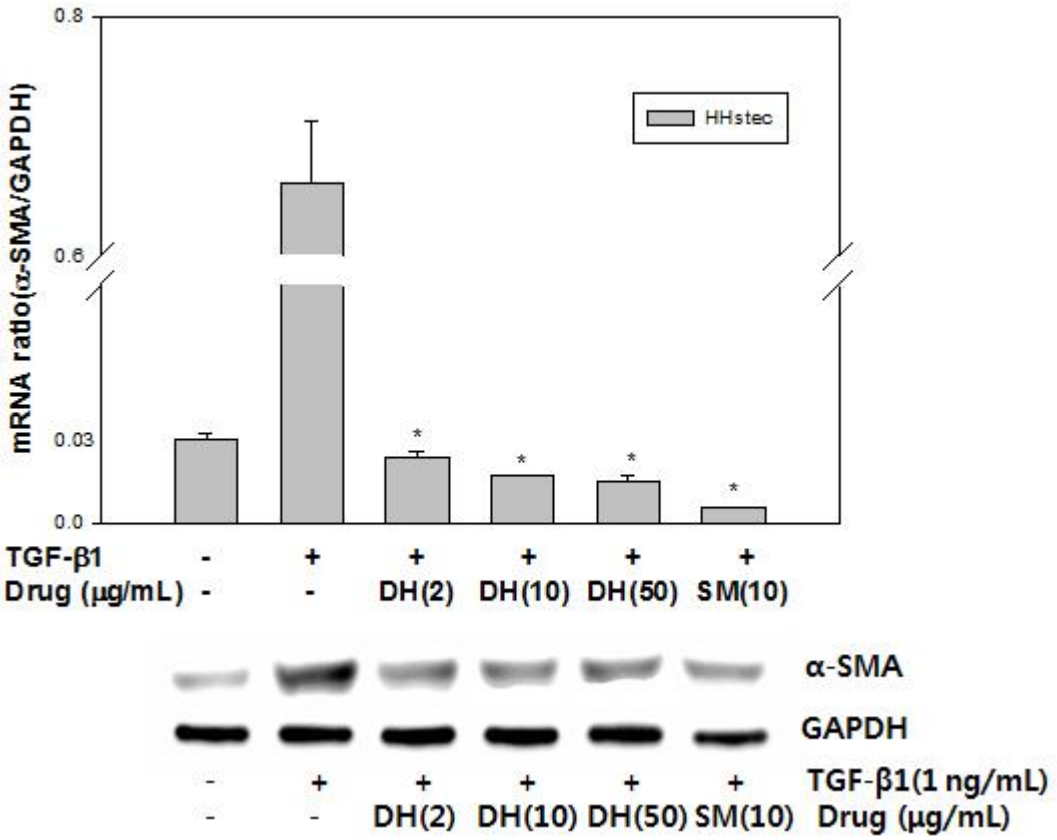

Fig. 2. Concentration-dependent effects of DMH on TGF-β1-induced α-SMA gene expression. SM, silymarin; *, P < 0.05.

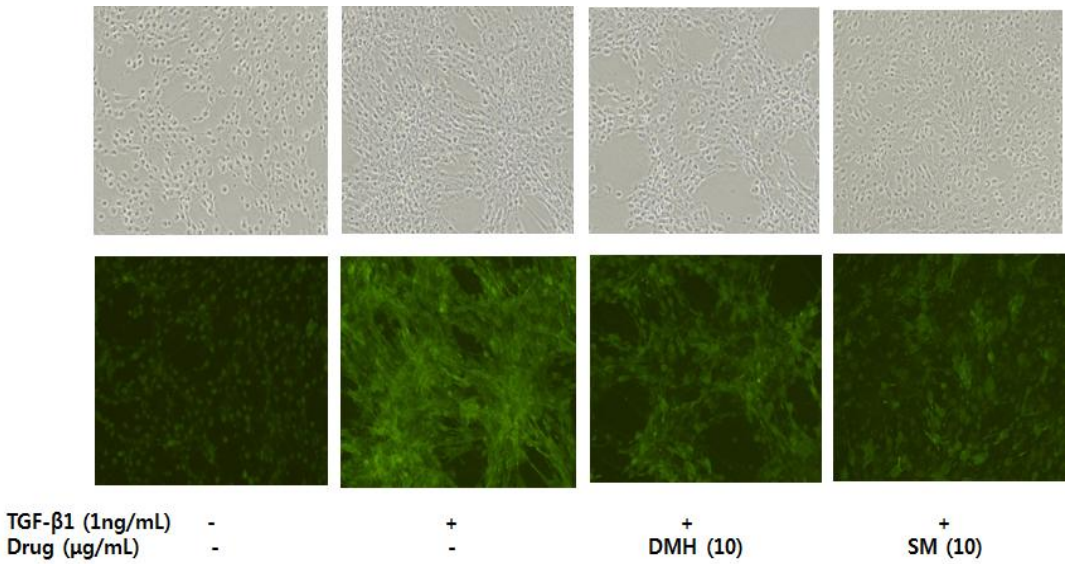

Fig. 3. Immunohistochemistry of TGF-β1-induced α-SMA expression after treatment of DMH and Silymarin in HSC.

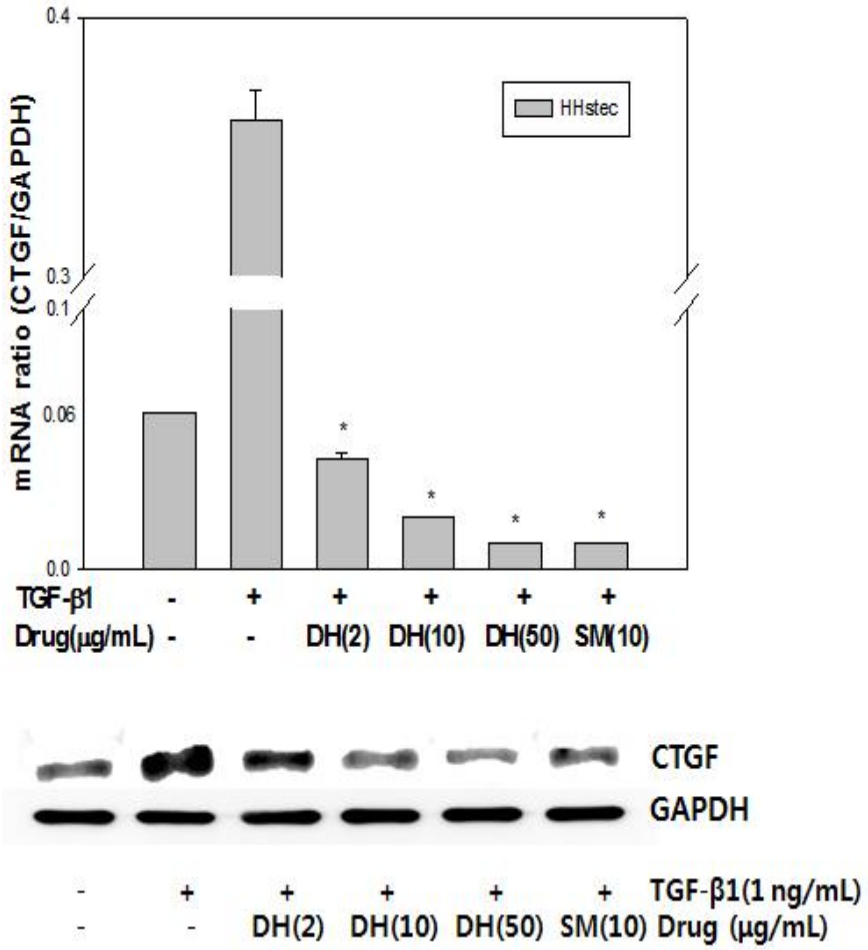

Fig. 4. Effect of DMH on TGF-β1-induced CTGF gene expression in HSC.

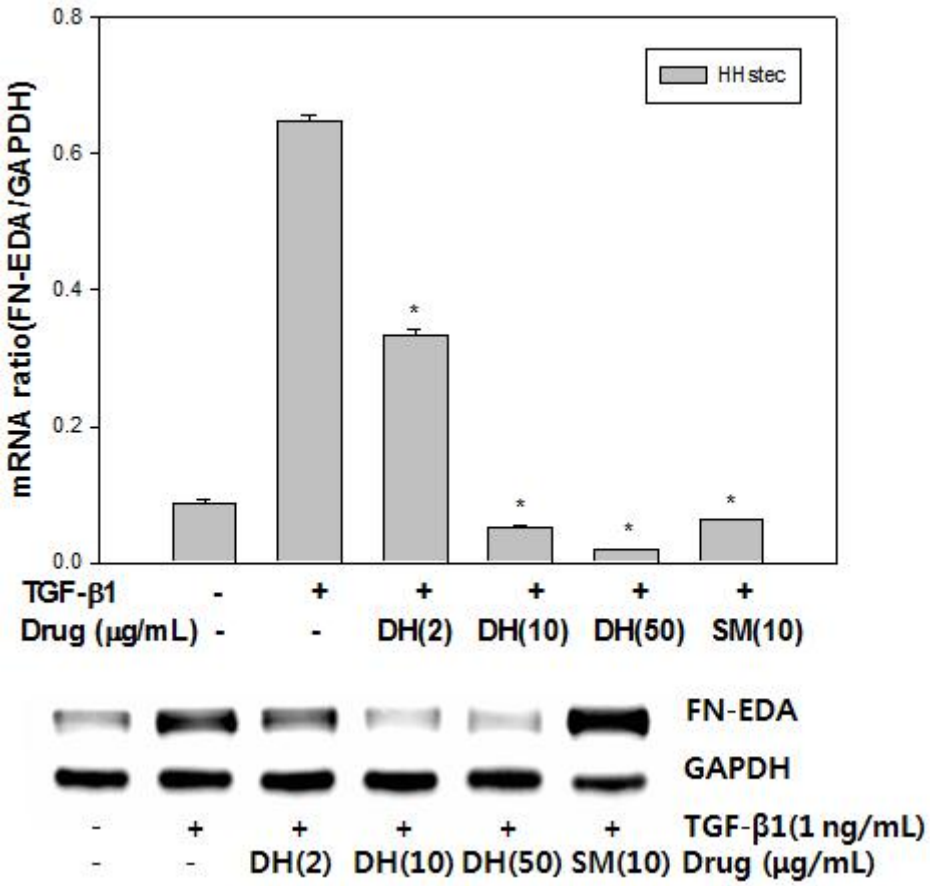

Fig. 5. Effect of DMH on TGF-β1-induced FN-EDA expression in HSC.

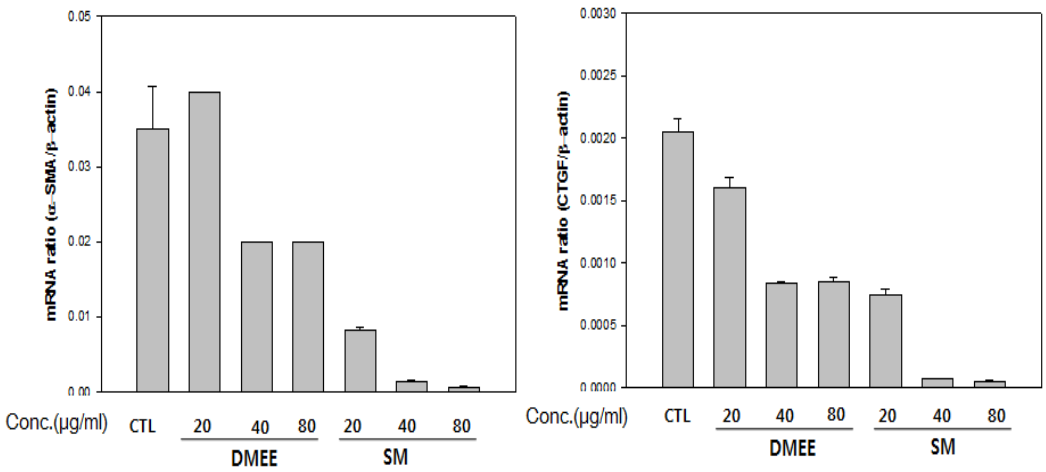

Fig. 6. Effect of DMEE on α-SMA and CTGF mRNA expression in HSCs.

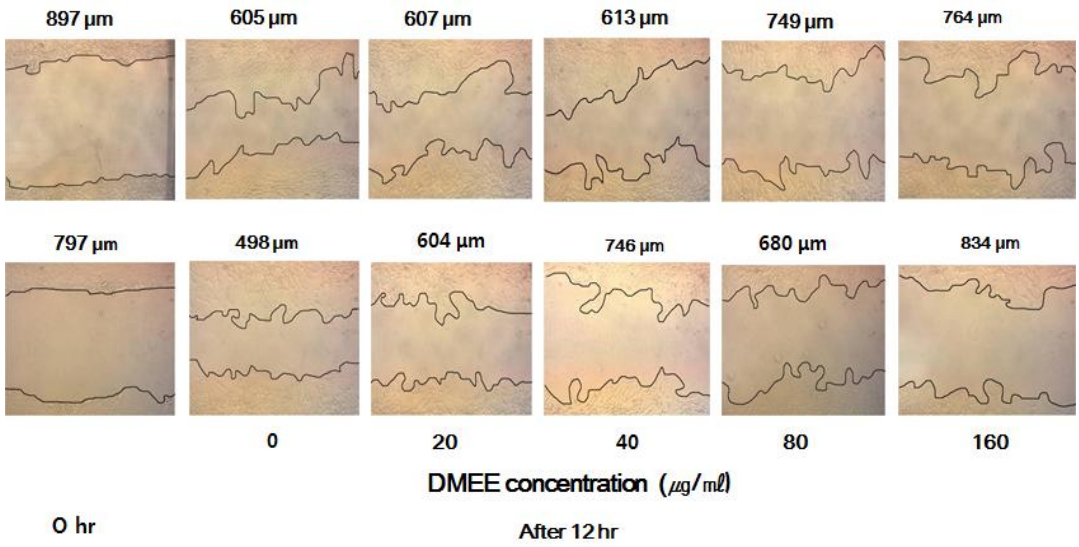

Fig. 7. Changes of scratch wound distance of HSCs after treatment of DMEE.

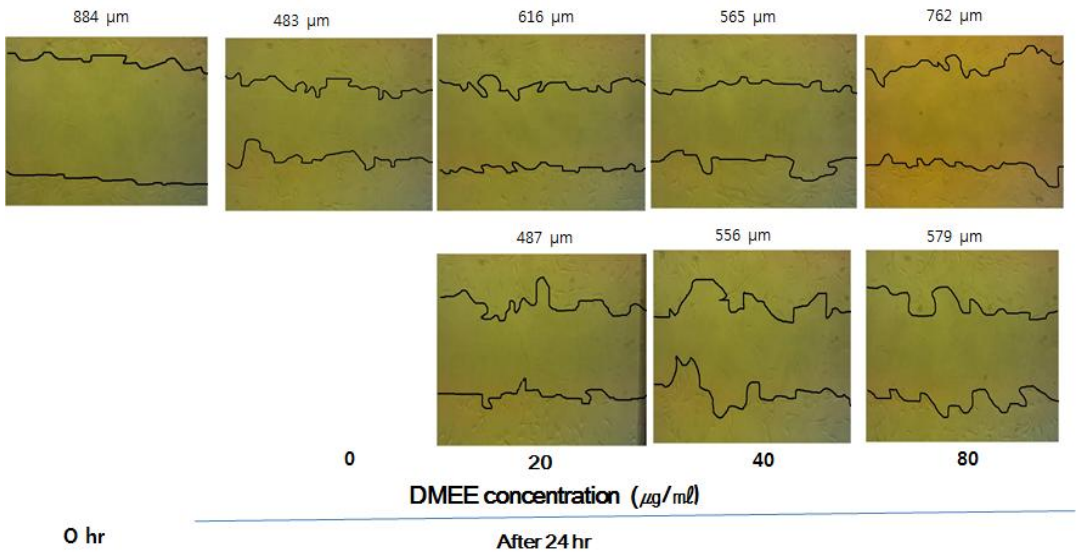

Fig. 8. Changes of scratch wound distance of Hs27 cells after treatment of DMEE.

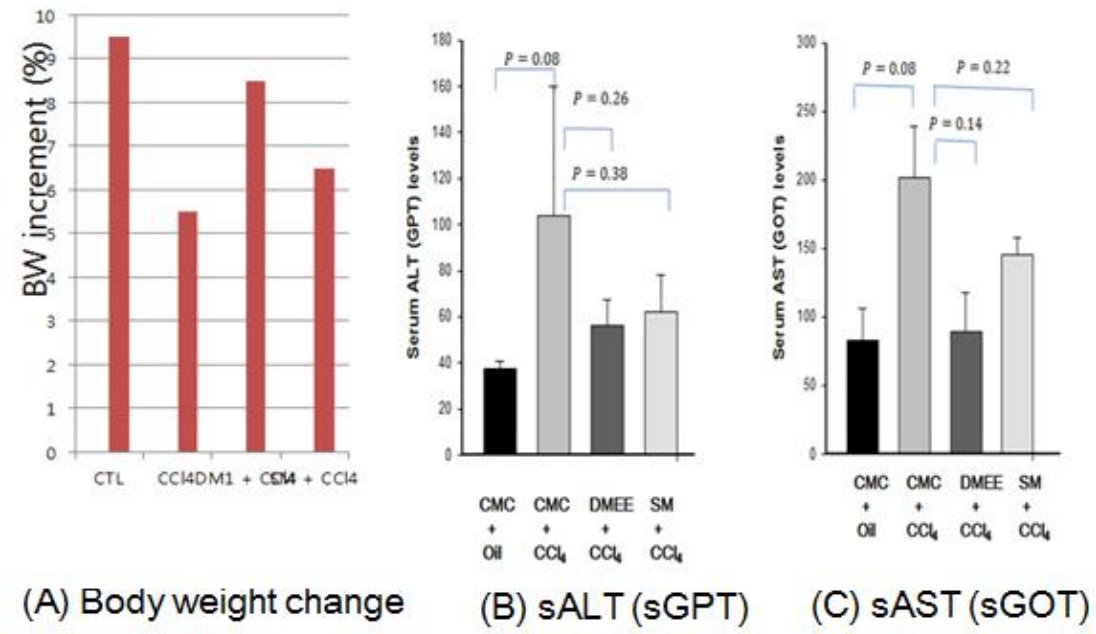

Fig. 9. Effects of oral pretreatment of DMEE or SM on body weight increment, serum ALT (sGPT) and serum AST (sGOT) levels in CCl_4 administered rats.

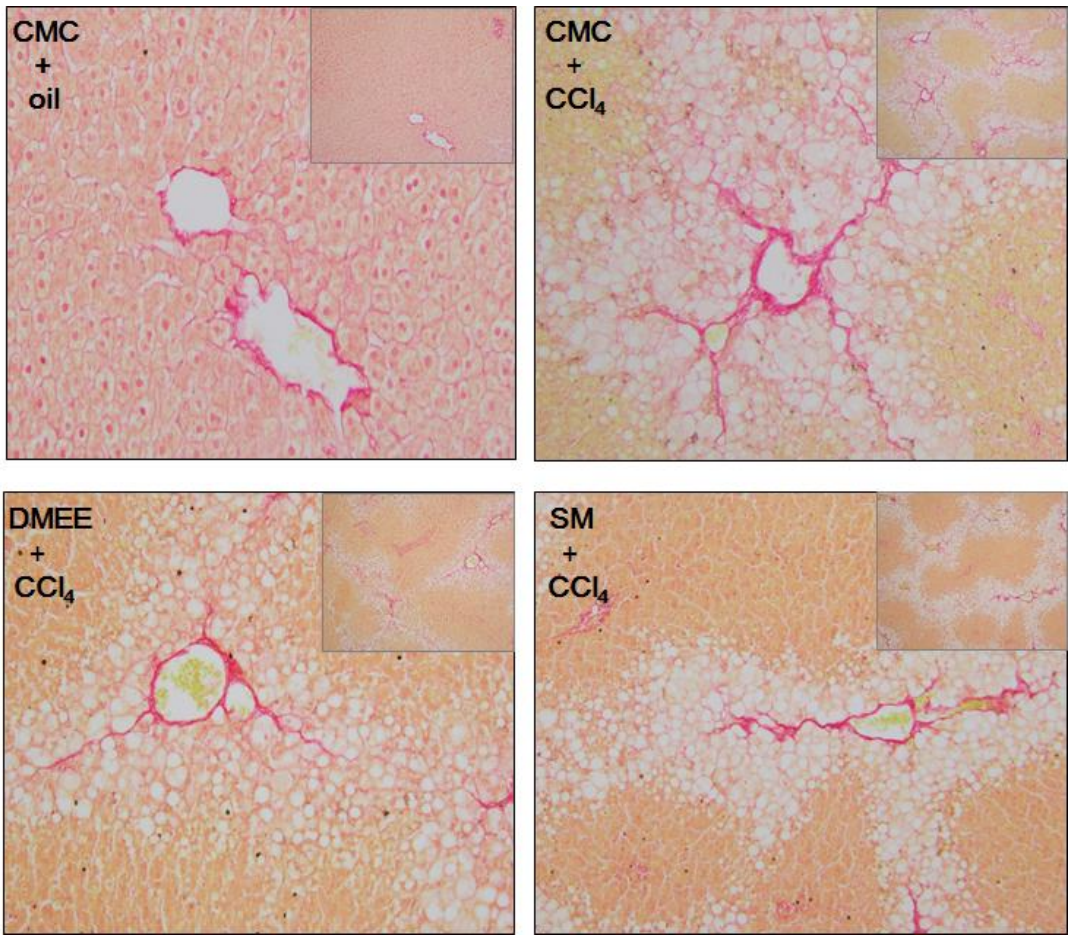

Fig. 10. Effects of oral pretreatment of DMEE of collagen accumulation in rat livers treated with CCl_4. CMC + oil: Fibrotic components such as collagen are limited in the central veins.CMC + CCl_4: Severe vacuolar changes of the hepatocytes and complete fibrotic septae interconnecting from central to central veins and from central to portal veins are observed; DMEE + CCl_4 and SM + CCl_4: Less degree of vacuolar changes of the hepatocytes than that observed in the CCl_4 only group and minute degree of radiating fibrotic bands around the vessel are noted. Inserts, lower magnifying power field.

Ⅳ. Abstract

Liver fibrosis inhibits liver function through increased tissue scarring. If left uncontrolled, it can progress into end-stage cirrhosis and/or hepatocellular carcinoma. Chronic liver injury caused by oxidative stress in hepatic stellate cells (HSCs) causes liver fibrosis by producing extracellular matrix proteins, such as collagen. In this study, *Dendropanax morbifera* (DM) extracts with strong antioxidant effects were investigated to assess their antifibrotic effects in the activated HSCs and CCl_4-induced rat liver fibrosis models. HSCs were activated with transforming growth factor-beta (TGF-β) and the expression of alpha-smooth muscle actin (α-SMA), connective tissue growth factor (CTGF), and fibronectin-extra domain A (FN-EDA) was measured using RT-PCR, Western blot analyses, or immunohistochemistry. Antioxidant effects of DM extracts were examined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay *in vitro* and 2',7'-dichlorofluorescin diacetate (DCFH-DA) assay in cells. Scratch-wound assays were performed to measure the migration of HSCs and Hs27 cells. Hepatoprotective and antifibrotic effects of DM ethanol extract (DMEE) extracted with 30% ethanol at 90℃ were compared with those of Silymarin (SM) in CCl_4-induced rat liver fibrosis model. Twenty-one Sprague-Dawley rats were divided into 4 groups: (1) vehicle (CMC and olive oil) group, (2) CMC and CCl_4 group,(3) oral DMEE pretreatment (100 mg/kg body weight) before CCl_4 group, and (4) oral Silymarin pretreatment (100 mg/kg/ body weight) before CCl_4 group. After administration twice a week for 6 weeks, all rats were sacrificed to prepare serum and liver samples for hepatotoxicity and histopathological examination. Blood samples were obtained from the abdominal vein. Body weight change and levels of ALT and AST were measured. For histological study, the tissues were stained with Sirius red for collagen I and III levels. Of the DM extracts, DM hexane extract (DMH) showed the strongest inhibitory activity for TGF-β1-induced α-SMA in HSCs. DMH also inhibited TGF-β1-induced α-SMA, CTGF, and FN-EDA mRNA and protein expression in a concentration-dependent mannerin

HSCs. Decreased expression of TGF-β1-induced α-SMA protein by DMH was confirmed by immunohistochemistry. DMEE has weaker antioxidant and antifibrotic activities but higher yield than DMH, suggesting its superiority with respect to safety and commercialization. DMEE inhibited the migration of fibroblasts such as HSCs and Hs27 cells. Pretreatment with DMEE or SM attenuated CCl_4-induced body weight decrease, elevation of serum AST and ALT, and collagen accumulation. Inconclusion, DM extracts can effectively inhibit liver fibrosis not only by inhibiting the activation and migration of HSCs *in vitro* but also by preventing hepatotoxicity and hepatic collagen accumulation *in vivo*. Thus, DM extracts with strong antioxidant effects may help prevent and treat liver fibrosis.

References

Aranda A, Sequedo L, Tolosa L, *et al*. (2013) Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay: a quantitative method for oxidative stress assessment of nanoparticle-treated cells. Toxicol In Vitro 27(2):954-63

Blom IE, Goldschmeding R, Leask A (2002) Gene regulation of connective tissue growth factor: new targets for antifibrotic therapy? Matrix Biol 21(6):473-82

Cory G (2011) Scratch-wound assay. Methods Mol Biol 769:25-30

Duval F, Moreno-Cuevas JE, Gonzalez-Garza MT, Rodriguez-Montalvo C, Cruz-Vega DE (2014) Protective mechanisms of medicinal plants targeting hepatic stellate cell activation and extracellular matrix deposition in liver fibrosis. Chin Med 9(1):27

Gebhardt R (2002) Oxidative stress, plant-derived antioxidants and liver fibrosis. Planta Med 68(4):289-96

Gressner OA, Gressner AM (2008) Connective tissue growth factor: a fibrogenic master switch in fibrotic liver diseases. Liver Int 28(8):1065-79

Hansen T, Christensen E (2015) [Cirrhosis and liver fibrosis are potentially reversible]. Ugeskr Laeger 177(48):V06150527

Hirabaru M, Mochizuki K, Takatsuki M, *et al*. (2014) Expression of alpha smooth muscle actin in living donor liver transplant recipients. World J Gastroenterol 20(22):7067-74

Kamei T, Asano K, Nakamura S (1986) Determination of serum glutamate

oxaloacetate transaminase and glutamate pyruvate transaminase by using L-glutamate oxidase. Chem Pharm Bull (Tokyo) 34(1):409-12

Kedare SB, Singh RP (2011) Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol 48(4):412-22

Kim H, Pan JH, Kim SH, Lee JH, Park JW (2018a) Chlorogenic acid ameliorates alcohol-induced liver injuries through scavenging reactive oxygen species. Biochimie 150:131-138

Kim HS, Lee TB, Choi CH (2001) Down-regulation of catalase gene expression in the doxorubicin-resistant AML subline AML-2/DX100. Biochem Biophys Res Commun 281(1):109-14

Kim S, Park SG, Song YJ, *et al*. (2018b) Analysis of Anticancer Activity and Chemical Sensitization Effects of Dendropanax morbifera and Commersonia bartramia Extracts. Anticancer Res 38(7):3853-3861

Kim W, Kim DW, Yoo DY, *et al*. (2015) Antioxidant effects of Dendropanax morbifera Leveille extract in the hippocampus of mercury-exposed rats. BMC Complement Altern Med 15:247

Kumar V, Mondal G, Dutta R, Mahato RI (2016) Co-delivery of small molecule hedgehog inhibitor and miRNA for treating liver fibrosis. Biomaterials 76:144-56

Luangmonkong T, Suriguga S, Mutsaers HAM, Groothuis GMM, Olinga P, Boersema M (2018) Targeting Oxidative Stress for the Treatment of Liver Fibrosis. Rev Physiol Biochem Pharmacol 175:71-102

Marques TG, Chaib E, da Fonseca JH, *et al*. (2012) Review of experimental models

for inducing hepatic cirrhosis by bile duct ligation and carbon tetrachloride injection. Acta Cir Bras 27(8):589-94

Moon HI (2011) Antidiabetic effects of dendropanoxide from leaves of Dendropanax morbifera Leveille in normal and streptozotocin-induced diabetic rats. Hum Exp Toxicol 30(8):870-5

Nakamura M, Ra JH, Kim JS (2016) The Comparative Analysis of Antioxidant and Biological Activity for the Dendropanax morbifera LEV. Leaves Extracted by Different Ethanol Concentrations. Yakugaku Zasshi 136(9):1285-96

Pan PH, Lin SY, Wang YY, *et al*. (2014) Protective effects of rutin on liver injury induced by biliary obstruction in rats. Free Radic Biol Med 73:106-16

Parajuli DR, Zhao YZ, Jin H, *et al*. (2015) Anti-fibrotic effect of PF2401-SF, a standardized fraction of Salvia miltiorrhiza, in thioacetamide-induced experimental rats liver fibrosis. Arch Pharm Res 38(4):549-55

Park BY, Min BS, Oh SR, *et al*. (2004) Isolation and anticomplement activity of compounds from Dendropanax morbifera. J Ethnopharmacol 90(2-3):403-8

Park SY, Karthivashan G, Ko HM, *et al*. (2018) Aqueous Extract of Dendropanax morbiferus Leaves Effectively Alleviated Neuroinflammation and Behavioral Impediments in MPTP-Induced Parkinson's Mouse Model. Oxid Med Cell Longev 2018:3175214

Sanchez-Valle V, Chavez-Tapia NC, Uribe M, Mendez-Sanchez N (2012) Role of oxidative stress and molecular changes in liver fibrosis: a review. Curr Med Chem 19(28):4850-60

Schleicher E, Wieland OH (1978) Evaluation of the Bradford method for protein determination in body fluids. J Clin Chem Clin Biochem 16(9):533-4

Seo JH, Kim JE, Shim JH, *et al*. (2016) HPLC Analysis, Optimization of Extraction Conditions and Biological Evaluation of Corylopsis coreana Uyeki Flos. Molecules 21(1):94

Shi H, Dong L, Jiang J, *et al*. (2013) Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway. Toxicology 303:107-14

Shi H, Shi A, Dong L, *et al*. (2016) Chlorogenic acid protects against liver fibrosis in vivo and in vitro through inhibition of oxidative stress. Clin Nutr 35(6):1366-1373

Shi HT, Shi AM, Shang BX, Wang Y, Dong L, Dai F (2015) [Effect of Chlorogenic Acid on Hepatic Stellate Cell Proliferation, Generation and Degradation of Extracelluar Matrix]. Sichuan Da Xue Xue Bao Yi Xue Ban 46(3):372-5

Song JH, Kang HB, Kim JH, *et al*. (2018) Antiobesity and Cholesterol-Lowering Effects of Dendropanax morbifera Water Extracts in Mouse 3T3-L1 Cells. J Med Food 21(8):793-800

Thiyagarajan V, Lee KW, Leong MK, Weng CF (2017) Potential natural mTOR inhibitors screened by in silico approach and suppress hepatic stellate cells activation.J Biomol Struct Dyn:1-15

Wight TN, Potter-Perigo S (2011) The extracellular matrix: an active or passive player in fibrosis? Am J Physiol Gastrointest Liver Physiol 301(6):G950-5

Xie WL, Jiang R, Shen XL, Chen ZY, Deng XM (2015) Diosgenin attenuates hepatic

stellate cell activation through transforming growth factor-beta/Smad signaling pathway. Int J Clin Exp Med 8(11):20323-9

감사의 글

먼저 제가 존경하는 스승님이시며 박사과정 동안 아낌없는 격려와 지도를 해주신 조수형 교수님께 감사드립니다. 그리고 저의 논문심사를 맡아주시고, 본 논문이 완성되기까지 세심한 지도와 많은 격려로 이끌어 주신 최철희 교수님께 진심으로 감사의 말씀 올립니다. 그리고 바쁘신 가운데 제 학위논문의 심사위원을 맡아주시고 좋은 조언을 해주신 김선표 교수님, 전영진 교수님, 전남대학교의 전병조 교수님께 감사드립니다.

평생 사랑으로 키워주시고 부족한 자식을 믿고 한결같이 지지하고 지원해주신 부모님께 감사의 말씀 드립니다. 부모님 덕분에 오늘의 제가 있을 수 있었습니다. 항상 저에게 격려와 응원을 아끼지 않는 장인어른과 장모님께 감사의 말씀을 드립니다.

항상 곁에서 든든한 버팀목이 되어주고, 힘이 되어주는 부인 정상인, 바람직하게 멋진 남자로 잘 자라주고 있는 아들 김효준, 김홍준에게 고마움과 사랑하는 마음을 전합니다.

마지막으로 지면을 통해 일일이 언급을 하지 못했지만 저를 아끼고 사랑해주신 모든 분들께 다시 한 번 진심으로 감사드립니다.