Thesis for Master's Degree

An exploratory study on anti-obesity and anti-MMP effects of the halophyte *Artemisia scoparia*

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요 약

현대의학의 발달로 인간의 수명은 계속해서 증가하고 있다. 그럼에도 불구하고 비만과 이에 따른 합병증, 암환자의 수는 매년 증가하고 있다. 지방전구세포인 3T3-L1는 지방생성 관련인자(C/EBPα, PPARɣ, SREBP1C) 활성화를 통하여 지방분화를 일으킨다. 또한 과잉 축적된 지방으로 인해 발생한 만성염증은 암과도 깊은 연관이 있는 것으로 알려져 있다. 특정 장기에 발생한 암세포는 다른 장기로까지 퍼지는 전이활동을 보인다. 기질금속단백질분해효소 (Matrix metalloproteinases, MMPs)는 세포외기질의 분해 및 재형성을 일으키는 아연의존성 효소이며, 암 또는 종양세포의 전이와 침윤에 관여한다. MMPs 중, 젤라틴 분해효소인 MMP-9과 MMP-2가 암 또는 종양세포의 전이와 침투에 주요한 역할을 한다. 천연소재로부터 이들의 활성을 저해하는 생리활성물질을 탐색하기 위해 염습지에 서식하는 염생식물을 선정하였다. 염생식물은 높은 염분을 포함하는 토양, 강한 바람, 직사광선 등과 같은 극한의 환경에서 생존하기 위해 육상식물과 차별된 생존전략을 가지고 있다. 따라서 육상생물과는 다른 이차대사산물이 존재할 것으로 사료된다. 실험에 사용된 염생식물 비쑥은 남•서해안에 자생하며 예로부터 민간전통의약으로 사용되어왔다. 또한 항산화, 항염증, 해열 및 진통, 살충효과가 있는 것으로 보고된바 있다.

서해안에 서식하는 비쑥을 채집한 후 dichloromethane과 methanol로 추출하여 조추출물을 얻었으며 이를 용매 극성도에 따라 분획하여 *n*-hexane, 85%

aqueous methanol (85% aq.MeOH), *n*-butanol (*n*-BuOH), water의 4가지 용매 분획물을 얻었다. 각각의 조추출물 또는 용매 분획물을 이용하여 항비만 관련 실험과 MMP-9과 MMP-2 억제 활성을 검토하였다. Oil Red O staining을 이용하여 각각의 용매 분획물에 의한 지방분화 정도를 확인하였다. 25 μg/mL의 85% aq.MeOH에 의해 유의적으로 지방분화가 억제 된 것을 확인할 수 있었다. 또한 지방생성 관련인자의 mRNA와 단백질 발현 또한 85% aq.MeOH에 의해 가장 효과적으로 억제 되었다. HT-1080 세포에서 MMPs의 효소적 활성도를 측정하기 위해 gelatin zymography를 실시하였고, *n*-hexane과 85% aq.MeOH 분획물이 MMP-9과 MMP-2의 효소적 활성도를 효과적으로 감소시켰다. Wound healing assay를 이용하여 비쑥 추출물 및 분획물이 세포이동에 미치는 영향을 측정하였을 때, 물 분획물을 제외한 모든 분획물에서 HT-1080 세포의 이동이 저해되는 경향을 보였다. Reverse transcription-polymerase chain reaction (RT-PCR)과 Western blot assay를 이용하여 MMP-9과 MMP-2의 mRNA와 단백질 발현 정도를 측정하였다. *n*-Heaxane 분획물이 MMP-9과 MMP-2의 mRNA 및 단백질의 발현을 가장 많이 저해였으며, 85% aq.MeOH 분획물도 유의한 mRNA와 단백질의 발현 억제효과를 보였다. 또한 *n*-BuOH 분획물에 의해 MMP-9과 MMP-2의 단백질 발현이 감소하는 것을 확인 할 수 있었다. Sandwich enzyme-linked immunosorbent assay (ELISA)를 통해 세포 외로 분비된 MMP-9과 MMP-2의 발현 정도를 측정하였는데, 분획물 중 *n*-hexane과 85% aq.MeOH 분획물이 MMP-9과 MMP-2의 발현을 가장 많이 억제하였다. MMP-2의 경우, *n*-BuOH과 물 분획물에 의해서도 발현이 감소한 것을 확인할 수 있었다. 항비만 및 MMP 활성억제 효과가 우수한 85% aq.MeOH 분획물에서 2차 대사물질 분리를 시도하였으며, Reynosin(**1**), Santamarine(**2**)을 분리하였다.

주제어: *Artemisia scoparia*; Preadipocytes (3T3-L1); Anti-obesity; Human fibrosarcoma cells (HT-1080); Matrix metalloproteinase (MMP); Reynosin; Santamarine

1. Introduction

1.1 Adipogenic differentiation

Many people in modern society are interested in diet and try to put it into practice. Nevertheless, the number of overweight or obese people is increasing. Obesity is believed to be caused by combination of several factors, including genetics, lack of exercise, and unhealthy and irregular eating habits. It is also known for obesity to cause diseases such as dementia, heart disease, liver disease, diabetes, arthritis, high blood pressure and cancer (Centers for Disease Control and Prevention, 2019). Preadipocytes are differentiated by factors such as insulin, isobutylmethylxanthin (IBMX) and dexamethasone to produce fats. The expression of CCAAT-enhancer binding proteins (C/EBPs) and peroxisome-proliferator activated receptors (PPARs), key transcription factors essential to adipogenic differentiation is increased - the transcription factors C/EBP β and C/EBP δ are activated by IBMX, which in turn activate PPAR γ and C/EBP α to induce adipocyte differentiation. Sterol regulatory element-binding protein 1c (SREBP1c) is a transcription factor that expresses genes related to cholesterol and fatty acid metabolism. It is regulated by insulin and expressed in early stage of differentiation process, and increases the transcriptional activity of PPAR γ (Valet et al., 2002) (Fig. 1).

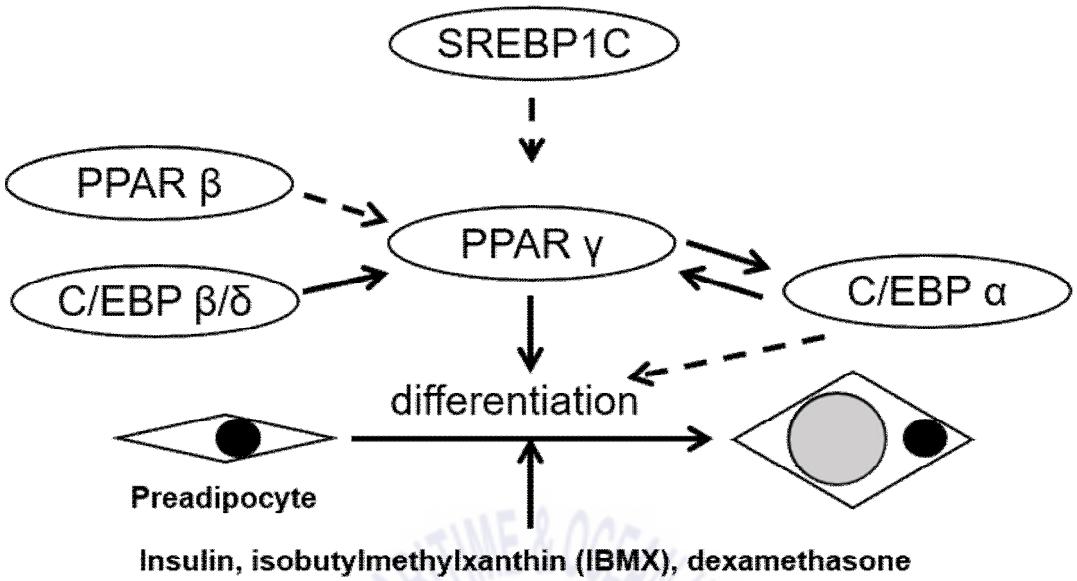


Fig. 1. Transcriptional process of adipogenic differentiation.

1.2 Metastasis and invasion of cancer cells

As the human lifespan has been prolonged with development of modern medicine, the incidience of various adult chronic diseases, including cancer, diabetes and neurodegenerative diseases rapidly has increased. Statistics Korea (KOSTAT, 2018) reported that cancer is the first cause of death in modern society of Korea and is one of issues which most people are most concerned for. According to the World Health Organization (WHO) Global Cancer Observatory statistics (2018), the incidence of cancer worldwide will increase by 63.1% until 2040. Also, National Cancer Center (NCC, 2018) reported that cancers have distant metastasis to other organs and have a survival rate of less than 10% in gastric cancer, lung cancer, liver cancer, biliary tract cancer, and pancreatic cancer. Matrix Metalloproteinases (MMPs) are involved in the proliferation and metastasis of cancer or malignant tumor cells. MMPs are zinc ion-dependent endopeptidases that are involved in the breakdown and remodeling of extracellular matrix (ECM) such as collagen, gelatin, elastin, matrix glycoproteins, and proteoglycans. (Verma & Hansch, 2007). MMPs are associated with physiological processes such as angiogenesis, apoptosis, bone remodeling, hair follicle circulation, immune response, and wound healing, as well as pathological progressions such as Alzheimer's, rheumatism, cardiovascular disease, skin ulcers, cirrhosis, and periodontal disease (Verma & Hansch, 2007). Expression of MMPs at the cellular level is regulated by inflammatory cytokines and growth factors secreted from cancer cells, and pro-MMPs (zymogen), inactive forms of MMP are secreted from host cells (Jabłońska-Trypuć et al., 2016). *In vivo*, MMPs are activated by other proteinases, *in vitro*, they can be activated by exposure to chemicals (Visse & Nagase, 2003). Activated MMPs are used for ECM degradation by attaching to cancer or tumor cells (Jabłońska-Trypuć et al., 2016). The MMPs that play key roles in the metastasis and invasion of cancer and tumor cells are

gelatinase A and B called MMP-2 (72 kDa) and MMP-9 (92 kDa) (Hannocks et al., 2017; McCawley & Matrisian, 2001). Expression of these MMPs is regulated by tissue inhibitors of MMPs (TIMPs, 21-29 kDa). TIMP-1 inhibits activity by specifically binding to pro MMP-9 while TIMP-2 does by binding to pro MMP-2. Thus, TIMPs perform an important role in balancing the collapse and reformation of ECM by MMPs (Fassina et al., 2000) (Fig. 2).

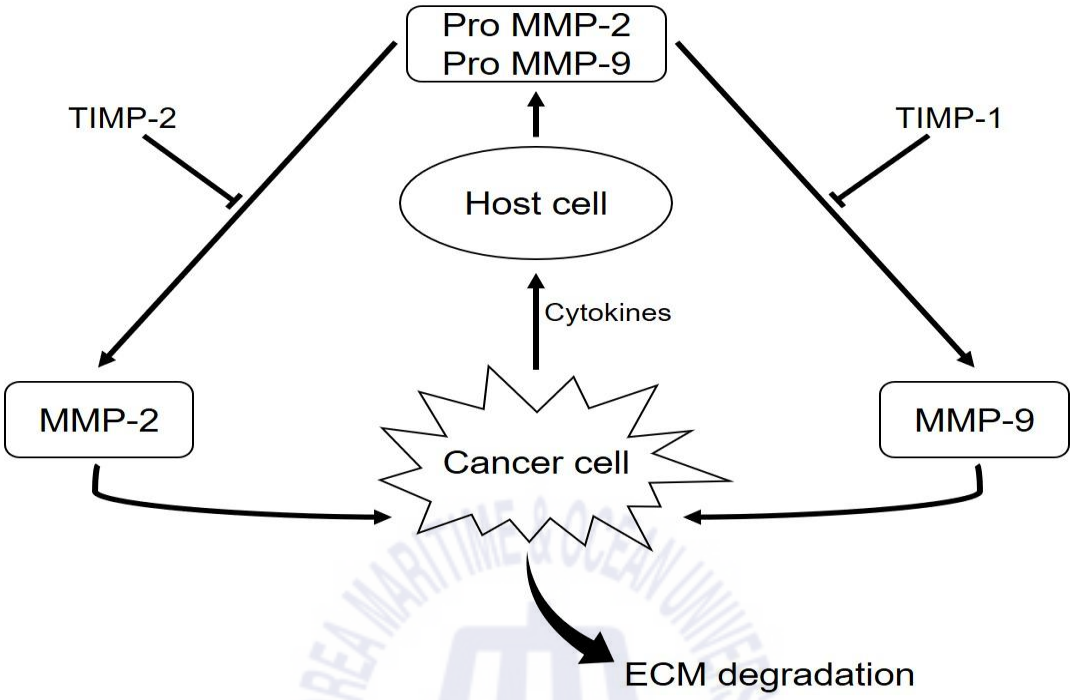


Fig. 2. Process of ECM degradation by MMP-2 and MMP-9.

1.3 The halophyte *Artemisia scoparia* Waldst. & Kit.

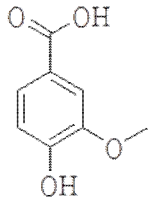
Since ancient times, mankind has used various kinds of herbs to treat various diseases, and has used them as herbal medicines. Over time, as terrestrial plants as source of new materials become increasingly depleted, research on marine organisms has been increasing. The ocean does not only occupy about 70% of the earth, but also more than 80% of the species on the earth live there. Since only about 1% of marine organisms have been chemically studied so far, there is a high probability of developing new drugs from the ocean.

Among these marine organisms, the halophyte is a salt-tolerant plant that grows in soil or waters of high salinity. It has an unique physiological mechanism for survival in harsh environments such as strong winds, sea spray, direct sunshine, and saline groundwater (Kim, 2016). Therefore, it is highly likely to isolate secondary metabolites with novel skeleton and unusual bioactivity from the halophytes. They are classified into two groups according to the distributing pattern - hydro-halophyte and xero-halophyte. The former is to grow in water or wet condition such as mangrove and salt marsh. The other is to grow on dry land and is succulent (Youssef, 2009). It is known that there are more than 2,500 species of halophytes all over the world (Ksouri et al., 2012). Korea, surrounded by the sea on three sides, has a well-developed halophyte habitat. About 100 species of salt marsh plants are distributed in coastal area of South Korea, including islands (Shim et al., 2017). Particularly, western coastal area of Korea is a good habitat of salt marsh plants because it has well-developed tidal flat (Shim et al., 2009).

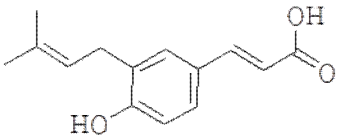
Artemisia scoparia Waldst. & Kit. is a variable plant with a life-cycle that can be annual, biennial or perennial and belongs to the genus *Artemisia*, family Asteraceae and order Asterales. This plant is native to Siberia or Caucasia in Russia, Mongol, India, China, Central Asia, Europe and entire region of Korea and grows on sandy ground of the beach. The plant produces much-branched stem in upper part which is 40-100 cm tall. Basal and lower stem leaves with stalks 1.5 cm long wither early. The middle and upper stem leaves are split into pinnate 2-3 times. The split leaves are thin like threads and become smaller and smaller as they go upwards. Flowers bloom in August to September and bear fruit in September to October.

A. scoparia has been used as a folk medicine for treatment of high fever, gout, jaundice, and urinary and skin diseases and its young sprout as a food (National Institute of Biological Resources). A variety of biological activities have bee reported for this halophyte: radical-scavenging, insecticidal, anti-nociceptive, anti-inflammatory, anticholesterolemic, antipyretic, antiseptic, antibacterial, diuretic, purgative and vasodilators (Singh et al., 2008; Negahban et al., 2006; Habib & Waheed, 2013). In addition, it has been used in the treatment of hepatitis, jaundice, and gall bladder inflammation (Him-Che, 1985). Fig. 3 shows secondary metabolites isolated from *A. scoparia* until now (Yahagi et al., 2014).

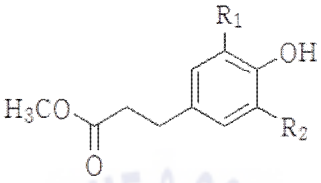
In this study, we investigated inhibitory effect of *A. scoparia* on adipogenesis and MMP activity using 3T3-L1 (preadipocyte) and HT-1080 (human fibrosarcoma cell).



Vanillic acid

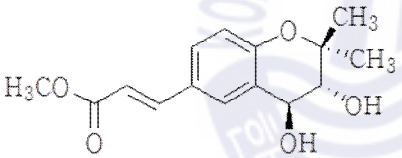


Drupanin

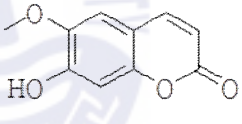


Methyl 3−(4'−hydroxyprenyl)coumarate : R_1= H, R_2= $CH_2CH{=}C(CH_3)CH_2OH$

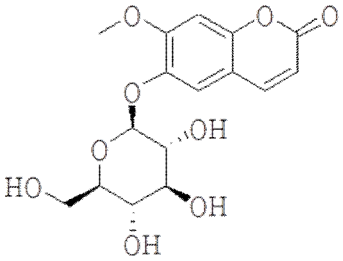
Methyl 1−(3',4'−dihydro−xyphenyl)propenoate : R_1= OH, R_2= H



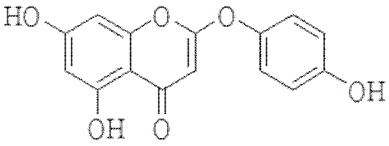
Scopariachromane



Scopoletin

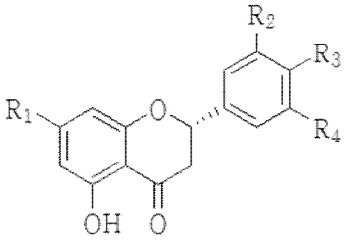


Magnolioside

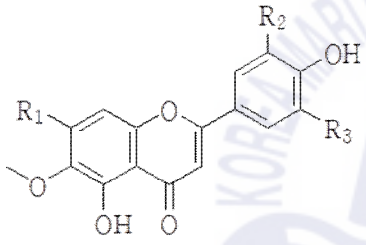


6−Demethoxycapillarisin

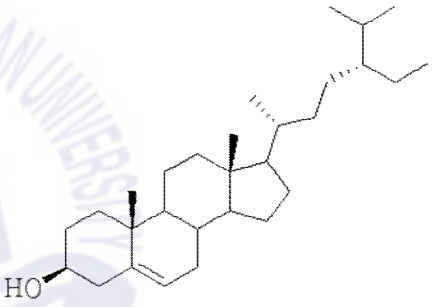
Fig. 3. Natural products isolated from *A. scoparia.*



Sakuranetin : R_1= OCH_3, R_2= H, R_3= OH, R_4= H
Naringenin : R_1= OH, R_2= H, R_3= OH, R_4= H
Blumeatin : R_1= OCH_3, R_2= OH, R_3= H, R_4= OH
3',5,5',7-Tetrahydroxyflavanone : R_1= OH, R_2= OH, R_3= H, R_4= OH
Eriodictyol : R_1= OH, R_2= OH, R_3= OH, R_4= H



Cirsiliol: R_1= CH_3O, R2= H, R_3= OH
Jaceosidin: R_1=OH, R_2= OCH_3, R_3= H



β-Sitosterol

Fig. 3. (continued)

2. Materials and Methods

2.1 Plant material

Whole plant of *Artemisia scoparia* was collected at Donggumdo in Gwanghwa-gun in September 2018.

Fig. 4. Photographs of the halophyte *A. scoparia*.

2.2 General experimental procedures

2.2.1 Reagents

Solvents used for extraction, fractionation and separation were first-class solvents purchased from DUKSAN (Korea) and SK chemicals (Korea). RP-18 (YMC-Gel ODS-A, 12 nm, S-75 μm) was used for the column packing materials, and Silica gel 60 F254 (1 mm, Merck) was used for the TLC plate. YMC pack ODS-A (250 × 10 mm, S 5 μm, 12 mm) was used as a column for HPLC, and a gaurd column (7.5 × 4.6 mm, Alltech) was used.

Dulbecco's modified eagle medium (DMEM), RPMI-1640 and Fetal Bovine Serum (FBS) used for cell culture were purchased from WELGENE (Korea), penicillin-streptomycin solution and 0.05% Trypsin-0.02% EDTA solution from Gibco (USA). PBS used for cell washing was purchased from BYLABS (Korea). Insulin, IBMX and dexamethasone in adipogenic differentiation media and Oil Red O solution were purchased from Sigma aldrich (USA). The phorbol 12-myristate 13-acetate (PMA) and RIPA buffer used to measure MMP expression were purchased from Sigma aldrich (USA). DuoSet ELISA kit, including capture antibodies (MMP-9, MMP-2) and detection antibodies (MMP-9, MMP-2) was purchased from R&D Systems Inc., (USA). Trizol was purchased from ambion life technologies™ (USA) and primers of PPARγ, C/EBPα, SREBP1C, MMP-9, MMP-2 and β-actin were purchased from Bioneer (USA). The primary and secondary antibodies of PPARγ, C/EBPα, SREBP1C, MMP-9, MMP-2 and β-actin used in Western blot assay were purchased from Cell signaling (USA).

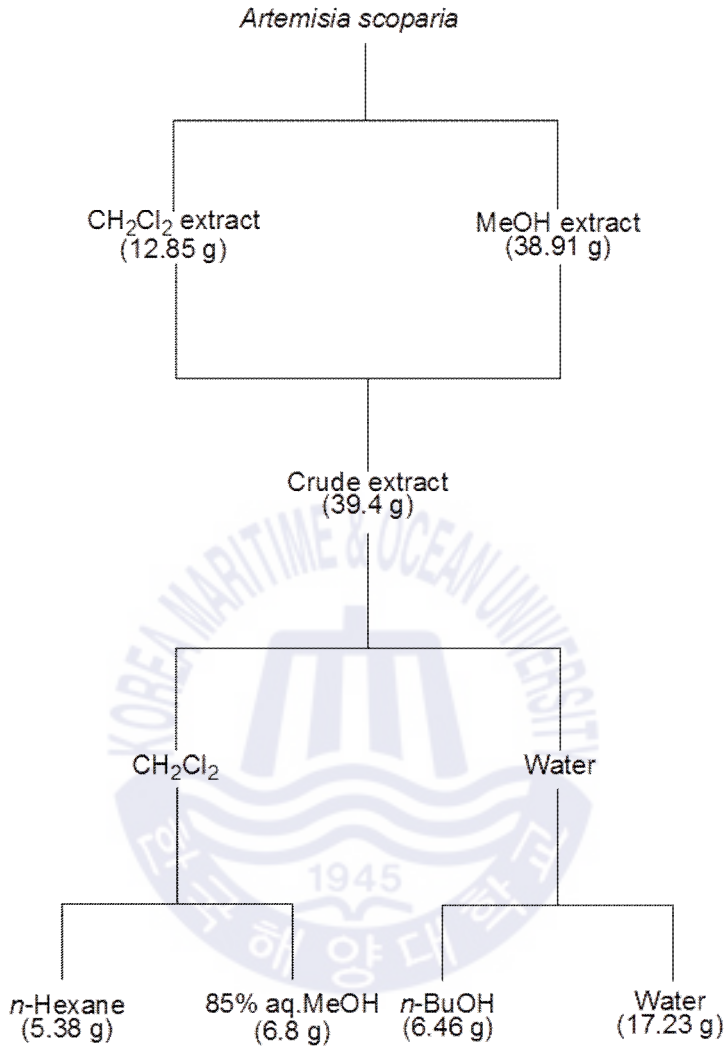
2.2.2 Experimental equipments

High performance liquid chromatography (HPLC) was performed on a Dionex P580 HPLC system equipped with a Varian 350 RI detector. All NMR experiments were performed on a Varian NMR 600 spectrometer (USA). UV-Vis spectrophotometer (Thermo Spectronic, England) and Victor3 1402 multilabel counter (Perkin Elmer, USA) were used to measure the absorbance of the MTT experiment. The thermal cycler used for PCR was purchased from Bio-rad (USA). Agarose and SDS-PAGE gels were image-developed using CAS-400SM Davinch-Chemi imager™ (Davinch-K, Korea).

2.3 Extraction, fractionation, and isolation

2.3.1 Extraction and fractionation

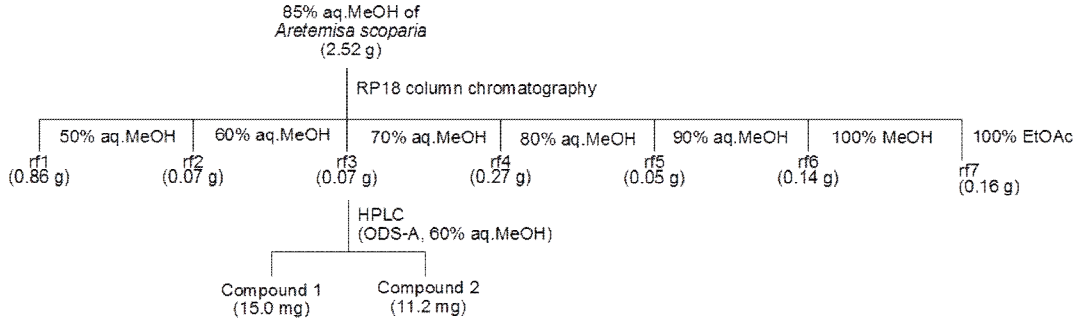
The collected *A. scoparia* was extracted twice with dichloromethane (CH_2Cl_2) and twice with methanol (MeOH) for 24 h, respectively. After desalting, combined crude extract (39.4 g) was fractionated with CH_2Cl_2 and water. The aqueous layer was further fractionated into *n*-butanol (*n*-BuOH) and water, and the organic layer was also fractionated into *n*-hexane and 85% aqueous MeOH (85% aq.MeOH), to afford *n*-hexane (5.38 g), 85% aq.MeOH (6.8 g), *n*-BuOH (6.46 g), water (17.23 g) fractions (Scheme 1).



Scheme 1. Preparation of crude extract and its solvent fractions from *A. scoparia*.

2.3.2 Isolation of compounds

A portion of 85% aq.MeOH fraction was divided into 7 fractions with C_{18} reverse phase vacuum flash chromatography [50%, 60%, 70%, 80%, 90% aq.MeOH, 100% MeOH, 100% ethyl acetate (EtOAc)] as shown in Scheme 2. Compounds **1** and **2** were separated from 70% aq.MeOH fraction by reversed-phase HPLC (YMC ODS-A, 2 ml/min, 60% aq.MeOH) (scheme 2).



Scheme 2. Isolation procedure of compound **1** and **2** from *A. scoparia*.

2.4 Anti-obesity activity

2.4.1 Culture of 3T3-L1 preadipocytes and their differentiation into adipocytes

3T3-L1 cells (preadipocytes) were distributed from ATCC and incubated in a 37℃, 5% CO_2 incubator with DMEM medium containing 10% FBS and 100 units/mL penicillin-streptomycin. Cultured cells were cultured by dispensing in 6 well plates to be 3.3×10^3 cells/well, and two days later, the cells were confluent and incubated for 48 hours in a medium containing 10% FBS, 5 μg/mL insulin, 0.25 μM dexamethasone, and 0.5 mM IBMX to induce differentiation. After 48 hours, differentiation was induced for 6 days by replacing the medium and samples containing only 10% FBS and 5 μg/mL insulin every two days.

2.4.2 Measurement of adipogenic differentiation in 3T3-L1 preadipocytes

Differentiation capacity of 3T3-L1 was measured by staining with Oil Red O. The culture medium of the differentiated cells was removed and washed with PBS, and then, treated with 10% formalin for 10 minutes for cell fixation. Intracellular fat was stained with Oil Red O solution for 30 minutes. After staining, the cells were washed with distilled water and the level of intracellular fat production was observed. After observation, distilled water was removed, dissolved in isopropyl alcohol, and the absorbance was measured at 500 nm.

2.4.3 Extraction of obesity-related mRNA and measurement of mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR)

The supernatant was removed and mRNA was extracted from the cells by adding Trizol reagent to the 3T3-L1 cells. After quantifying the extracted mRNA to 2 μg, RNase-free water and oligo dT were added, denaturated at 70℃ for 5 minutes and maintained at 4℃. A mixture containing 1X RT buffer, 1 mM dNTP, 500 ng oligo dT 140 U M-MLV reverse transcriptase and 40 U RNase inhibitor was added and reverse transcribed using a thermal cycler for 1 hour at 42℃ and 5 minutes at 72℃. β-Actin, a house keeping gene, was used as internal control. Each target DNA was amplified by adding the PPARγ, C/EBPα, SREBP1C and β-actin primers.

Target DNA amplification was performed predenaturation at 94℃ for 5 min, followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at 60℃ for 1 min and extension at 72℃ for 1 min. It was held at 72℃ for 5 min and then terminated at 4℃.

The synthesized DNA product was electrophoresed at 100 mV for 10 minutes in 1% agarose gel. Gel was stained in 1 mg/mL EtBr solution and photographed under UV light using CAS-400SM Davinch-Chemi imager™.

Table 1. Primer sequences of obesity-related genes and β-actin

Gene name	Direction	Sequence
PPAR γ	Forward	5′-TTT-TCA-AGG-GTG-CCA-GTT-TC-3′
	Reverse	5′-AAT-CCT-TGG-CCC-TCT-GAG-AT-3′
C/EBP α	Forward	5′-TTA-CAA-CAG-GCC-AGG-TTT-CC-3′
	Reverse	5′-GGC-TGG-CGA-CAT-ACA-GTA-CA-3′
SREBP1C	Forward	5′-TGT-TGG-CAT-CCT-GCT-ATC-TG-3′
	Reverse	5′-AGG-GAA-AGC-TTT-GGG-GTC-TA-3′
β-actin	Forward	5′-AGC-CAT-GTA-CGT-AGC-CAT-CC-3′
	Reverse	5′-TCC-CTC-TCA-GCT-GTG-GTG-GT-3′

2.4.4 Measurement of obesity-related protein levels by Western blot assay

Protein was extracted from the cells using RIPA buffer to the 3T3-L1 cells. The extracted protein was quantified by Bradford protein assay, Quantitative proteins (20 μg) were loaded on 12% sodium dodecylslufate-polyacrylamide gel and electrophoresed at 100 V for 90 minutes. Proteins were transferred from the gel to nitrocellulose transfer membrane (Whatman®, UK). The transferred membrane was blocked with 5% skim milk in 1X TBST, and then incubated at 4℃ for 24 hours by adding a primary antibody diluted 1: 4000 in 1X TBST. (PPAR γ, C/EBP α, SREBP1C, β-actin). After that, the primary antibody was removed and washed with 1X TBST. After incubating for 1 hour with a secondary antibody, Western blotting detection reagent kit (GE Healthcare, UK) was sprayed onto the membrane to confirm protein band through CAS-400SM Davinch-Chemi imager™

2.5 Matrix metalloproteinase inhibitory activity

2.5.1 HT-1080 cells culture

HT-1080 cells (human fibrosarcoma cells) were distributed from Korea Cell Line Bank (KCLB) and used for experiments. Cell culture was performed using RPMI-1640 medium containing 100 units/mL penicillin-streptomycin and 10% FBS. Cells were incubated in 37℃, 5% CO_2 incubator, washed with 1X PBS buffer 4-5 times a week and then the medium was replaced. Attached cells were detached using 0.05% Trypsin-0.02% EDTA solution and subsequently passaged.

2.5.2 Measurement of cell viability by MTT assay

To determine the effect of inhibiting cell proliferation on substances obtained from the *A. scoparia*, cytotoxicity to HT-1080 cells was measured using MTT [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide] reagent.

Cells were incubated in a 96 well plate at 37℃, 5% CO_2 incubator for 24 hours at a density of 5×10^3 cells/well. After replacement with fresh medium, each sample was treated by concentration. After 24 hours, cultured cells were treated with 1 mg/mL MTT reagent and incubated for 4 hours to induce formazan formation by mitochondrial enzymes. After removing the MTT reagent, After dissolving formazan by adding 100 μL DMSO, the absorbance was measured at 540 nm using a Victor3 multilabel plate reader. Relative cell viability was determined by the amount of MTT converted into a formazan. Viability of cells was quantified as a percentage compared to the control.

$$\text{Cytotoxicity}(\%) = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

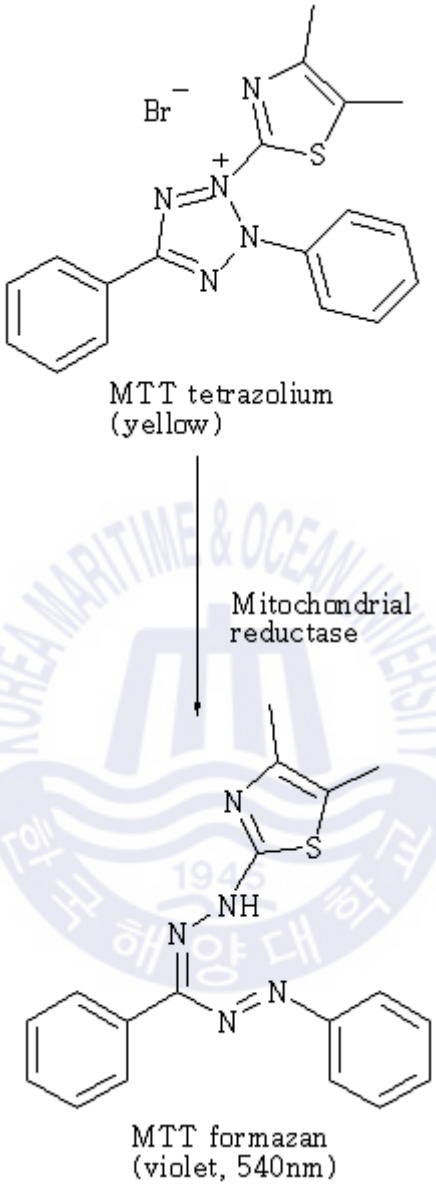


Fig. 5. Metabolization of MTT to a MTT formazan by viable cells.

2.5.3 Measurement of MMP activity by gelatin zymography

Gelatin zymography was used to measure the enzymatic activity of MMP-9 and MMP-2 secreted by HT-1080 cells.

HT-1080 cells were incubated for 24 hours at 37℃, 5% CO_2 conditions in a 24 well plate to be 2×10^5 cells/well. After replacement with serum-free medium, the prepared samples were processed by concentration. After 1 hour, PMA (10 ng/mL) was treated to induce MMPs expression. In order to confirm the total protein amount of the supernatant obtained by stimulation for 24 hours, Bradford protein quantification was performed to include the same amount of protein. Quantitative proteins were electrophoresed on 10% sodium dodecylsulfate-polyacrylamide gel containing 1.5 mg/mL gelatin. After electrophoresis, the polyacrylamide gel was washed for 30 minutes with a buffer containing 50% Triton X-100 (50 mM Tris-HCl, pH 7.5). In order to induce gelatin hydrolysis by MMP, it was incubated for 48 hours at 37 ℃ in developing buffer (10 mM CaCl 2, 50 mM Tris-HCl, 150 mM NaCl). The gel was stained with Commssie brilliant blue 250 for 30 minutes and destained with a destaining solution (acetic acid: MeOH: water = 10: 50: 40). The transparent bands of gelatin hydrolyzed by MMP were photographed using CAS-400SM Davinch-Chemi imager™. The area of the transparent band was measured and then graphed.

2.5.4 Measurement of protein levels by enzyme-linked immunosorbent assay (ELISA)

Changes in the amount of secretion of MMP-9 and MMP-2 by extracts and fractions of *A. scoparia* in PMA-induced HT-1080 cells were measured using sandwich ELISA. 100 μL of capture antibodies (MMP-9: 1 μg/mL, MMP-2: 2 μg/mL) were attached to each well of a 96 well plate overnight at room temperature. After washing three times with 25X Wash buffer (0.05% Tween 20 in PBS) diluted to 1X, 300mL 10X Reagent diluent (1% BSA in PBS) diluted to 1X was treated and blocked for 1 hour. After washing three times with 1X Wash buffer, 100 μL of a constant concentration of the samples were treated and reacted at room temperature for 2 hours. The supernatants prepared for gelatin zymography were used as samples. After washing three times with 1X Wash buffer, each well was treated with 100 μL of detection antibody (MMP-9: 150 ng/mL, MMP-2: 10 ng/mL) and reacted at room temperature for 2 hours. After washing three times with 1X Wash buffer, Streptavidin-HRP (horseradish-peroxidase) was treated and reacted in the dark for 20 minutes. After washing three times with 1X Wash buffer, the 100 μL of Substrate solution (tetramethylbenzidine : H_2O_2 = 1 : 1) was treated and reacted in the dark for 20 minutes. After adding 50 mL of Stop solution (2 NH_2SO_4), the absorbance was measured with a Victor3 multilabel plate reader at 450 nm.

2.5.5 Cell migration observation using the wound healing assay

HT-1080 cells were grown in a 12 well plate for 24 hours at 37℃ and 5% CO_2 so that the cell density per well is 80－90%. Using a sterile plastic tip, draw a 2 mm wide injury line in the center of each well. After remove the floating cells by washing with 1X PBS buffer, exchanged with fresh serum-free medium. In each well, samples were treated at a concentration of 100 μg/mL. Cell migration was observed with an inverted microscope and photographs were taken immediately after the samples were treated and when 24 hours had elapsed.

2.5.6 mRNA extraction and measurement of mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR)

HT-1080 cells were incubated for 24 hours at 37℃ and 5% CO_2 in 6 well plates to be 1×10^4 cells/well. After replacement with serum-free medium, the prepared samples were processed by concentration. After 1 hour, PMA (10 ng/mL) was treated to induce MMP expression. After 24 hours, the supernatant was removed and mRNA was extracted from the cells by adding Trizol reagent to the remaining cells. After quantifying the extracted mRNA to 2 μg, RNase-free water and oligo dT were added, denaturated at 70℃ for 5 minutes and maintained at 4℃. A mixture containing 1X RT buffer, 1 mM dNTP, 500 ng oligo dT 140 U M-MLV reverse transcriptase and 40 U RNase inhibitor was added and reverse transcribed using a thermal cycler for 1 hour at 42℃ and 5 minutes at 72℃. Each target DNA was amplified by adding the MMP-2, MMP-9 and β-actin primers.

Target DNA amplification was performed predenaturearation at 95℃ for 2 min, followed by 34 cycles of denaturation at 95℃ for 30 sec, annealing at 60℃ for 45 sec and extension at 72℃ for 1 min.

The synthesized DNA product was electrophoresed at 100 mV for 10 minutes in 1.5% agarose gel. Gel was stained in 1 mg/mL EtBr solution and photographed under UV light using CAS-400SM Davinch-Chemi imager™.

Table 2. Primer sequences of MMP-2, MMP-9 and β-actin

Gene name	Direction	Sequence
MMP-2	Forward	5′ -ATG-GCA-AGT-ACG-GCT-TCT-GT-3′
	Reverse	5′ -ATA-CTT-CTT-GTC-GCG-GTC-GT-3′
MMP-9	Forward	5′ -CTC-GAA-CTT-TGA-CAG-CGA-CA-3′
	Reverse	5′ -GCC-ATT-CAC-GTC-GTC-CTT-AT-3′
β-actin	Forward	5′ -AGC-CAT-GTA-CGT-AGC-CAT-CC-3′
	Reverse	5′ -TCC-CTC-TCA-GCT-GTG-GTG-GT-3′

2.5.7 Measurement of protein levels by Western blot assay

Western blot experiments were performed to measure the expression of MMP at the protein level. HT-1080 cells were cultured and samples were treated in the same manner as RT-PCR described above, and then protein was extracted from the cells using RIPA buffer. The extracted protein was quantified by Bradford protein assay, Quantitative proteins (20 μg) were loaded on 12% sodium dodecylslufate-polyacrylamide gel and electrophoresed at 100 V for 90 minutes. Proteins were transferred from the gel to nitrocellulose transfer membrane (Whatman®, UK). The transferred membrane was blocked with 5% skim milk in 1X TBST, and then incubated at 4℃ for 24 hours by adding a primary antibody diluted 1: 4000 in 1X TBST. (MMP-9, MMP-2, β-actin). After that, the primary antibody was removed and washed with 1X TBST. After incubating for 1 hour with a secondary antibody, western blotting detection reagent kit (GE Healthcare, UK) was sprayed onto the membrane to confirm protein band through CAS-400SM Davinch-Chemi imager™.

2.6 Statistical analysis

The significance test of the experimental results was carried out by ANOVA and Duncan's multiple range test at $p < 0.05$ level, and the results were expressed as mean ± standard deviation. All statistical analyzes were performed using the statistic analysis system v.9.1 (SAS Institute Inc., USA) statistical program.

3. Result and Discussion

3.1 Structural determination of compounds isolated from *A. scoparia*

Compound **1** was obtained as an amorphous white solid. The ^{1}H NMR spectrum of **1** showed signals for a tertiary methyl (δ 0.80, s), two oxymethines (δ 4.10, t, J = 10.9 Hz; δ 3.49, dd, J = 11.6, 4.5 Hz), and two exomethylenes [(δ 6.00, d, J = 3.1 Hz; δ 5.49, d, J = 3.0 Hz) and 4.95, s; δ 4.79, s)]. The ^{13}C NMR and HSQC spectra exhibited the presence of 15 carbons, including one ester carbonyl carbon, four olefinic carbons (two methylenes and two quaternary carbons), two oxymethine carbons, eight aliphatic carbons (one methyl, four methylenes, two methines, and one quaternary carbon). Particularly, in ^{1}H and ^{13}C NMR spectra, signals at δ_H 6.00 (1H, d, J=3.1 Hz), δ_H 5.49 (1H, d, J=3.0 Hz), δ_H 4.10 (1H, t, J=10.9 Hz), δ_C 171.7, δ_C 141.3, δ_C 117.3, and δ_C 81.5 indicated the presence of α-exomethylene-γ-lactone. HMBC correlations of δ_H 6.00 (H-13) and 5.49 (H-13) with δ_C 171.7 (C-12), and δ_H 5.49 (H-13) and δ_C 141.3 (C-11) supported this interpretation. By literature survey based on the above mentioned information, **1** was identified as reynosin which was previously isolated from *Laurus nobilis*, *Costus speciosus*, *Diaspananthus uniflorus*, and *Magnolia grandiflora*. The NMR spectral data for **1** were in good agreement with those reported in the literature (Fang et al., 2005; Al-Attas et al., 2015; Adegawa et al., 1987; El-Felary & Chan, 1978).

Compound **2** was isolated as an amorphous white solid. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to those obtained for **1**. However, there appeared significant differences in the ^{13}H NMR spectrum. Signals of olefinic methine and methyl appeared at δ 5.36 (1H, m, H-3) and 1.81 (3H, s, H-15), respectively while those of the exomethylene protons at δ 4. 95 (1H, s, H-15) and 4.79 (1H, s, H-15) in **1** disappeared. Corresponding changes were found in the ^{13}C NMR spectrum in which signals of new olefinic methine and methyl carbons were observed at δ 122.8 and 22.1, respectively instead of

exomethylene carbon signal of **1**, suggesting that the double bond of C-4 and C-15 of **1** migrated to other position. The new position of the transferred double bond was confirmed to be between C-3 and C-4 by the extensive 2D experiments such as ^{1}H-^{1}H COSY, HSQC, and HMBC. Literature survey revealed that this compound was santamarine, the known compound which was in a good accordance with spectral data reported previously (Fang et al., 2005; Al-Attas et al., 2015). To the best of our knowledge, compounds **1** and **2** were the first isolation from the genus *Artemisia*.

Table 3. ^{1}H and ^{13}C NMR spectral data for compound **1** isolated from *A. scoparia*

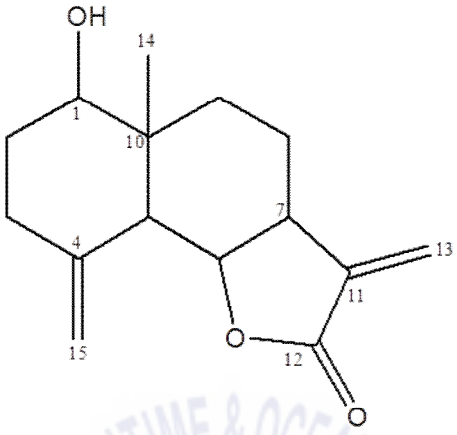
Position	δ_H	δ_C
1	3.49 (1H, dd, J=11.6, 4.5 Hz)	78.8
2	1.80, 1.56 (1H, m)	32.1
3	2.32 (1H, ddd, J=13.5, 4.9, 1.8 Hz), 2.15 (1H, td, J=13.5, 5.1)	34.8
4		145.1
5	2.24 (1H, br d, J=10.9 Hz)	54.1
6	4.10 (1H, t, J=10.9 Hz)	81.5
7	2.61 (1H, tdd, J= 11.2, 3.1, 3.0 Hz)	50.7
8	2.08, 1.60 (1H, m)	22.4
9	2.05 (1H, m), 1.38 (1H, td, J=13.3, 3.8)	36.9
10		44.2
11		141.3
12		171.7
13	6.00 (1H, d, J=3.1 Hz), 5.49 (1H, d, J=3.0 Hz)	117.3
14	0.80 (3H, s)	12.0
15	4.95, 4.79 (1H, s)	110.2

Measured in CD_3OD at 600 and 151 MHz, respectively. Assignments were aided by 1H COSY, gHSQC and gHMBC experiments.

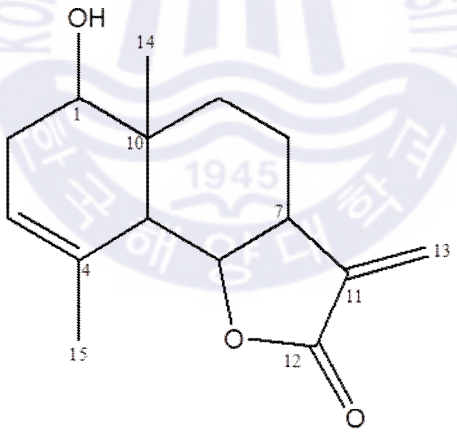
Table 4. ^{1}H and ^{13}C NMR spectral data for compound **2** isolated from *A. scoparia*

Position	δ_H	δ_C
1	3.61 (1H, dd, J=10.1, 6.7 Hz)	75.8
2	2.32, 1.97 (1H, m)	33.5
3		122.8
4		134.6
5	2.39 (1H, d, J=11.1 Hz)	52.4
6	4.01 (1H, t, J=11.1 Hz)	83.5
7	2.57 (1H, tdd, J= 11.2, 3.1, 3.0 Hz)	52.2
8	2.09, 1.66 (1H, m)	22.1
9	2.02 (1H, dt, J=13.2, 3.4), 1.33 (1H, td, J=13.2, 3.3 Hz)	35.6
10		42.2
11		141.0
12		172.8
13	6.00 (1H, d, J=3.2 Hz), 5.36 (1H, d, J=3.1 Hz)	117.1
14	0.87 (3H, s)	11.4
15	1.81 (3H, s)	23.6

Measured in CD_3OD at 600 and 151 MHz, respectively. Assignments were aided by 1H COSY, gHSQC and gHMBC experiments.



Compound 1



Compound 2

Fig. 6. Chemical structure of compounds **1** and **2** from *A. scoparia*.

3.2 Anti-obesitic effect of solvent fractions from crude extract of *A. scoparia*

3.2.1 Effect of solvent fractions on lipid accumulation in 3T3-L1 adipocytes

In order to confirm the effect of the solvent fractions on the fat globules produced in the differentiated 3T3-L1 cells, the fat globules were identified using Oil Red O solution staining. Samples were treated at 25, 10, 1 μg/mL concentrations, respectively. The percentage of fat accumulation was expressed based on the control untreated with samples. As shown in Fig. 7, all solvent fractions inhibited formation of lipid droplets in 3T3-L1 adipocytes, compared with the control. Among them, the 85% aq.MeOH fraction showed the strongest inhibition effect at concentration of 25 μg/mL (Fig. 8).

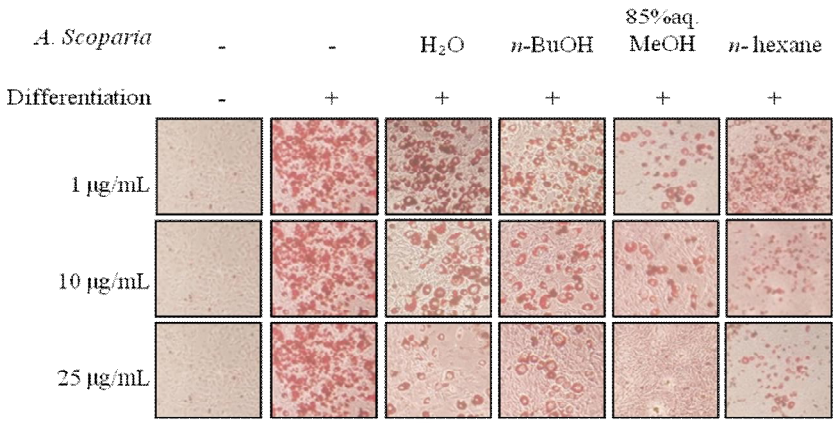


Fig. 7. Observation of lipid droplets stained with Oil Red O in 3T3-L1 adipocytes.

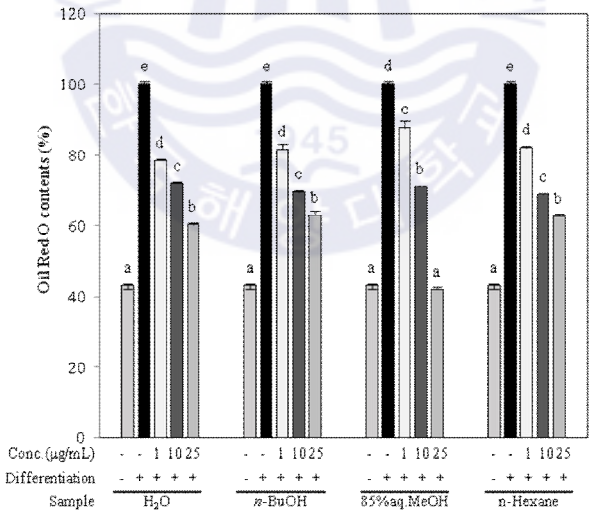


Fig. 8. Effect of solvent fractions from *A. scoparia* on fat accumulation in 3T3-L1 adipocytes by Oil Red O staining. $^{a-e}$Means with different letters are significantly different ($p < 0.05$) by Duncan's multiple range test. Values are means ± SD.

3.2.2 Effect of solvent fractions on mRNA levels of obesity-related genes by RT-PCR

Reverse transcription PCR analysis were conducted to estimate the effect of solvent fractions on mRNA expression of PPAR γ, SREBP1C and C/EBP α, key transcription factors during adipogenesis.

The *n*-hexane fraction reduced significantly mRNA expression level of SREBP1C at the contration of 25 μg/mL while the 85% aq.MeOH fraction suppressed mRNA expression levels of PPAR γ and C/EBP α. Particulary, the 85% aq.MeOH fraction inhibited potenly mRNA expression level of C/EBP α even at the contration of 10 μg/mL (Fig. 9).

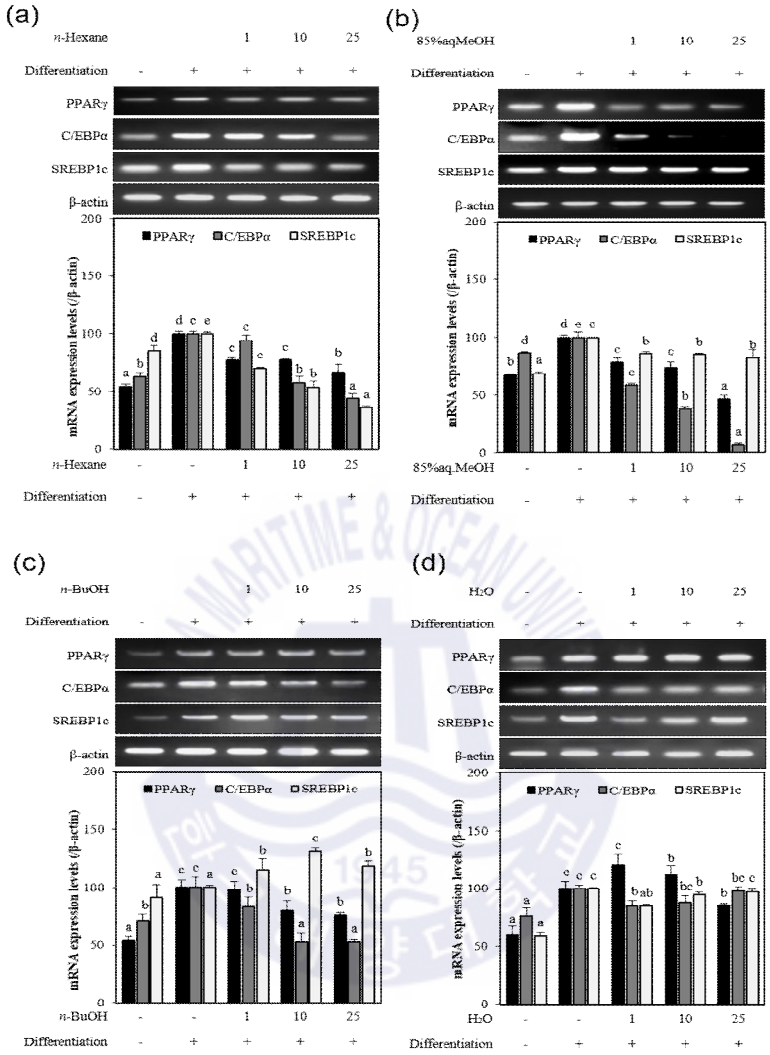


Fig. 9. Effect of solvent fractions from *A. scoparia* on mRNA expression levels of obesity-related genes determined by RT-PCR. β-Actin was used as an internal standard. Band sizes were calculated and depicted as percentage compared to control group. Values were normalized against house keeping β-actin mRNA levels. Values are means ± SD. $^{a-c}$Means with different letters at the same concentration are significantly different ($p<0.05$) by Duncan's multiple range test. (a) *n*-hexane, (b)85 aq.MeOH, (c) *n*-BuOH and (d) water.

3.2.3 Effect of solvent fractions on protein levels of obesity-related transcription factors by Western blot assay

During adipogenic differentiation, inhibitory effect of solvent fractions on protein expression levels of key transcription factors PPAR γ, SREBP1C, and C/EBP α was analyzed by Western blot assay. When differentiation-induced cells were treated with the solvent fractions, the protein expression levels of PPAR γ, C/EBP α and SREBP1C were decreased in a dose-dependent manner by all fractions except water faction. The 85% aq.MeOH fraction exhibited the most potent inhibition effect against key transcription factors, followed by the *n*-BuOH and *n*-hexane fractions as shown in Fig. 10.

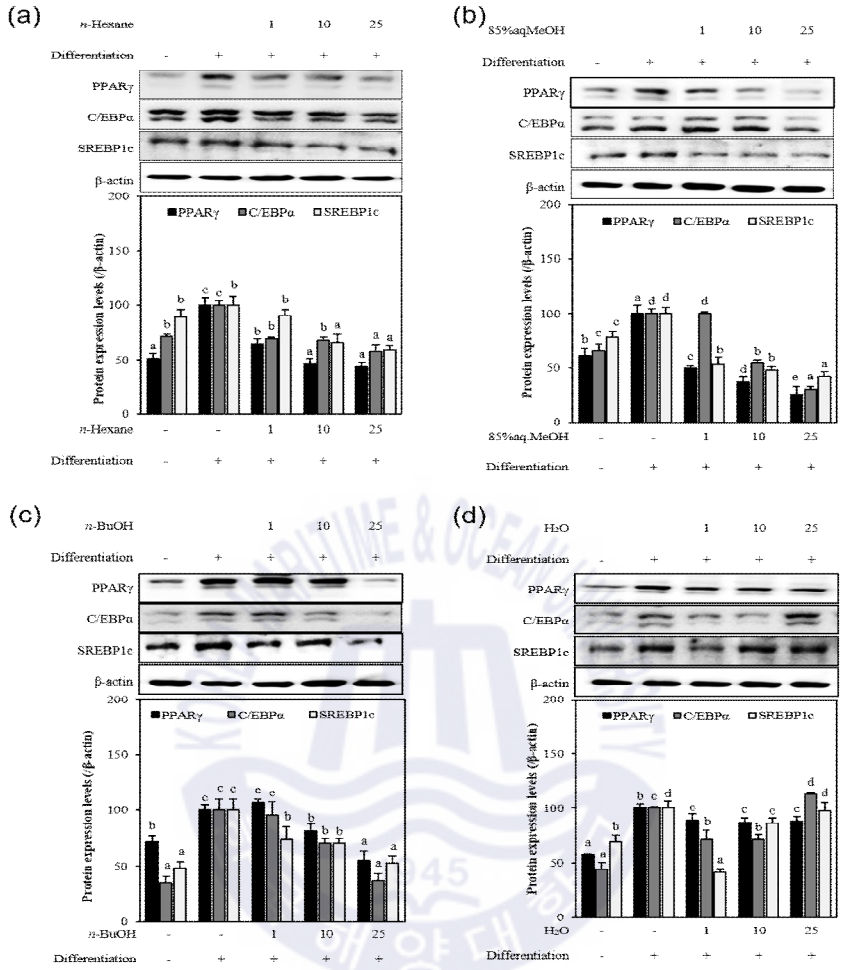


Fig. 10. Effect of solvent fractions from *A. scoparia* on protein levels of obesity-related genes determined by Western blot assay. β-Actin was used as an internal standard. Band sizes were calculated and depicted as percentage compared to control group. Values were normalized against house keeping β-actin protein levels. Values are means ± SD. $^{a-d}$Means with different letters at the same concentration are significantly different ($p<0.05$) by Duncan's multiple range test. (a) *n*-hexane, (b)85 aq.MeOH, (c) *n*-BuOH and (d) water.

3.3 Effects of crude extract and its solvent fractions from *A. scoparia* on MMP activity

3.3.1 Effects of crude extract and its solvent fractions on cell viability of HT-1080 cells

Cytotoxicity of crude extract and its solvent fractions on HT-1080 cells was measured at concentrations of 200, 100, 50 and 10 μg/mL, respectively, to determine cell viability. All samples showed cytotoxicity at 200 μg/mL concentration, and cell viability was under 70% at 100 μg/mL concentration of all samples except *n*-BuOH fraction. At 50 μg/mL concentration, treatment of all samples except *n*-hexane fraction (cell viability: 72%) showed cell viability of more than 80%. Therefore, evaluation for inhibitory effect of the samples on MMPs was carried out at 50 μg/mL (Fig. 11).

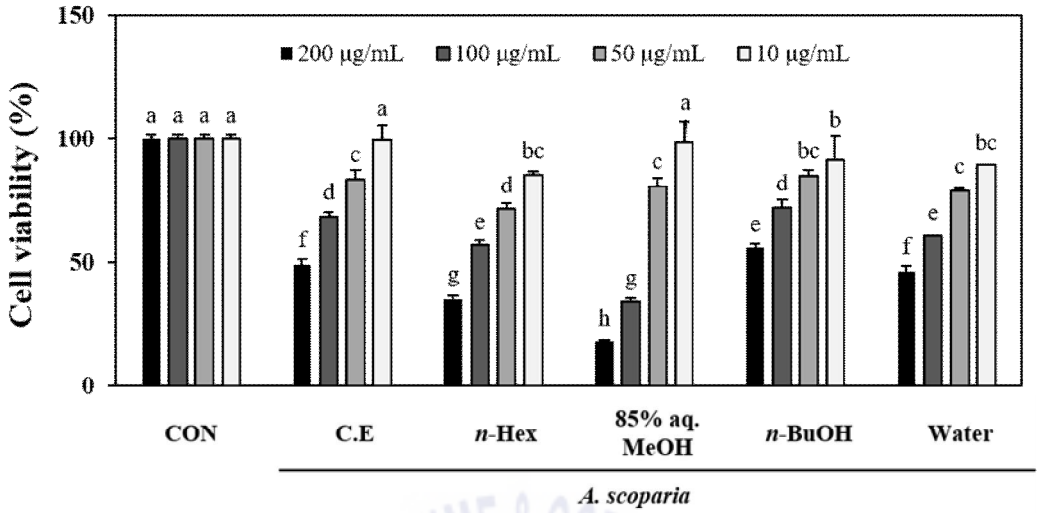


Fig. 11. Effects of crude extract and its solvent fractions from *A. scoparia* on cell viability of HT-1080 cells. [a-h]Means with different letters are significantly different ($p < 0.05$) by Duncan's multiple range test. Values are means ± SD.

3.3.2 Effect of crude extract and its solvent fractions on enzymatic activity of MMP-9 and 2 by gelatin zymography

Gelatin zymography was performed to determine the enzymatic activity of MMP-9 and MMP-2 secreted into the supernatant of the cultured HT-1080 cell. Expression levels of MMP-9 and MMP-2 were significantly increased in only PMA-treated cells. When cells were treated together with the sample and PMA, 50 μg/mL concentration of crude extract reduced expressions of MMP-9 and MMP-2 to 60.4% and 41.7%, respectively, compared to the control.

Among the solvent fractions, the *n*-hexane fraction decreased expression levels of MMP-9 and MMP-2 to the largest rates, 72.2% and 54.0%, respectively. The 85% aq.MeOH fraction suppressed expression levels of MMP-9 and MMP-2 by 58% and 43.7%. Compared to control and blank, *n*-hexane and 85% aq.MeOH fractions showed almost complete inhibition rate at 50 μg/mL concentration. *n*-BuOH and water fractions revealed little inhibitory effects on expressions of MMP-9 and MMP-2 (Fig. 12).

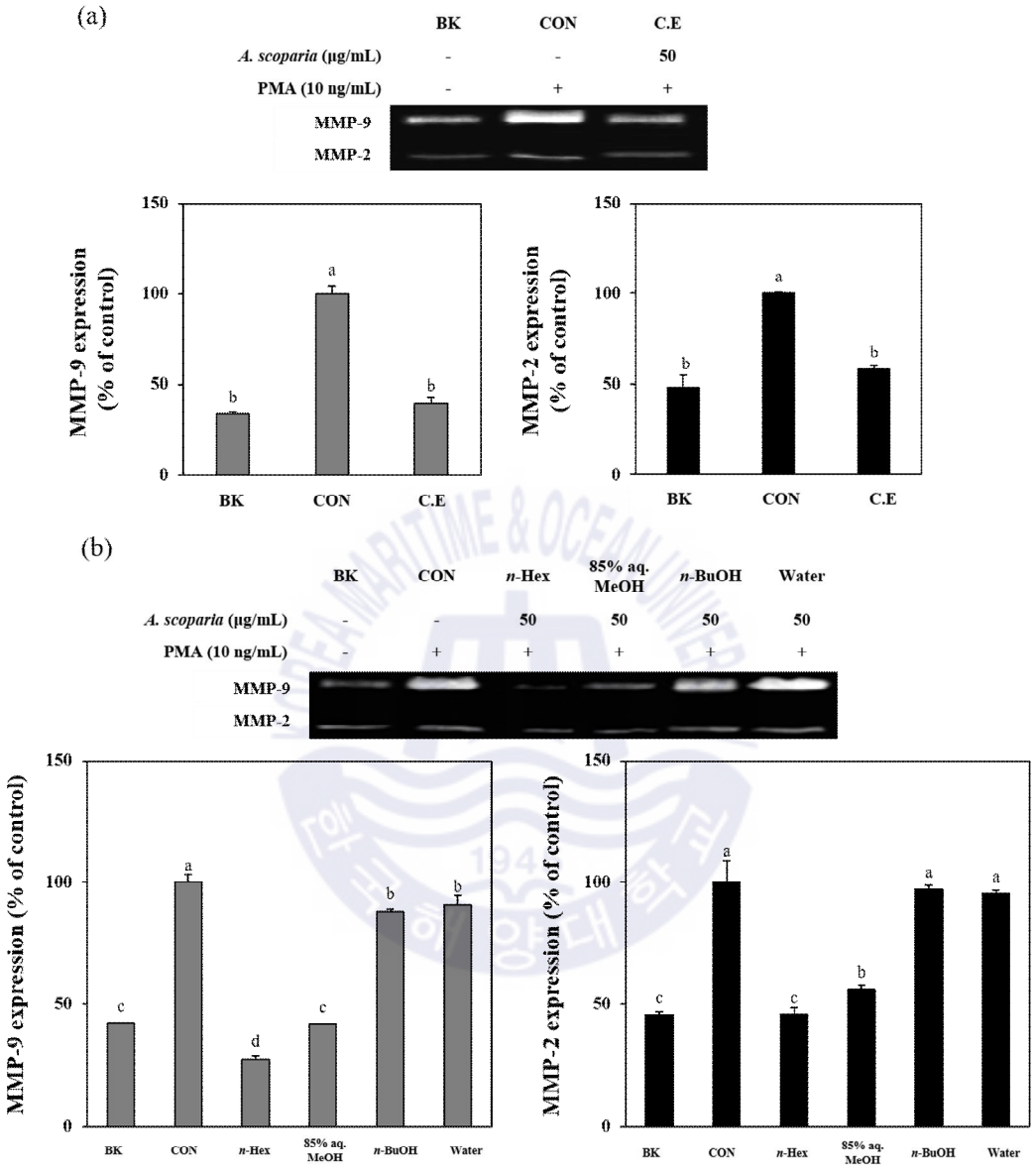


Fig. 12. Effect of crude extract (a) and its solvent fractions (b) from *A. scoparia* on enzymatic activity of MMP-9 and 2 determined by gelatin zymography in HT-1080 cells. a-dMeans with different letters are significantly different ($p < 0.05$) by Duncan's multiple range test. Values are means ± SD.

3.3.3 Effect of crude extract and its solvent fractions on released protein levels of MMP-9 and 2 by ELISA

Sandwich ELISA was performed to evaluate the inhibitory effect of crude extract and its solvent fractions on protein levels of MMP-9 and MMP-2 released from PMA-stimulated HT-1080 cells. In this method, MMP-2 and MMP-9 proteins in culture supernatants were analyzed by ELISA kit. After PMA stimulation of HT-1080 cells, the cells were incubated in the presence of each of crude extract and its solvent fractions for 24 h.

Among them, *n*-hexane and 85% aq.MeOH fractions most effectively inhibited secretion of MMP-9 and MMP-2 at concentration of 50 μg/mL. Secretions of MMP-9 and MMP-2 were reduced to 356.1 pg/mL and 0.52 ng/mL respectively, by *n*-hexane fraction, and 398.5 pg/mL and 0.53 ng/mL respectively, by 85% aq.MeOH fraction (Fig. 13).

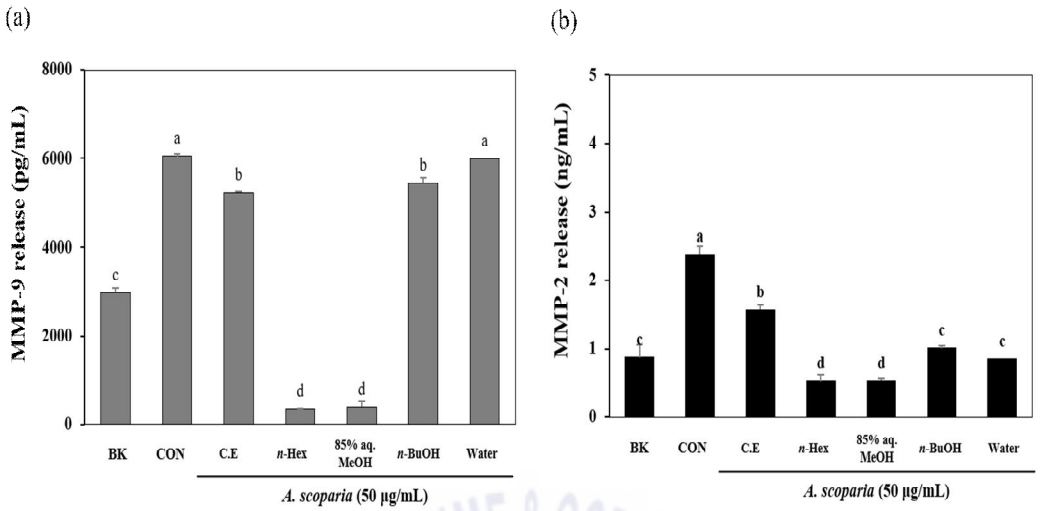


Fig. 13. Effect of crude extract and its solvent fractions from *A. scoparia* on released protein levels of MMP-9 (a) and 2 (b) determined by sandwich ELISA in HT-1080 cells. $^{a-e}$Means with different letters are significantly different ($p < 0.05$) by Duncan's multiple range test. Values are means ± SD.

3.3.4 Effect of crude extract and its solvent fractions on cell migration ability by the wound healing assay

MMPs, especially MMP-2, are known to be involved in cell migration and invasion (Okoli, 2014). Therefore, The patterns of cell migration affected by sample treatment were examined through wound healing assay. Cells not treated with the sample (control) showed an obvious migration after 24 hours, while the cells treated with it did that migration was suppressed. Among samples tested, the water fraction little exhibited the inhibitory effect on cell migration. However, crude extract, *n*-hexane, 85% aq.MeOH and *n*-BuOH fractions were effective in inhibiting cell migration (Fig. 14).

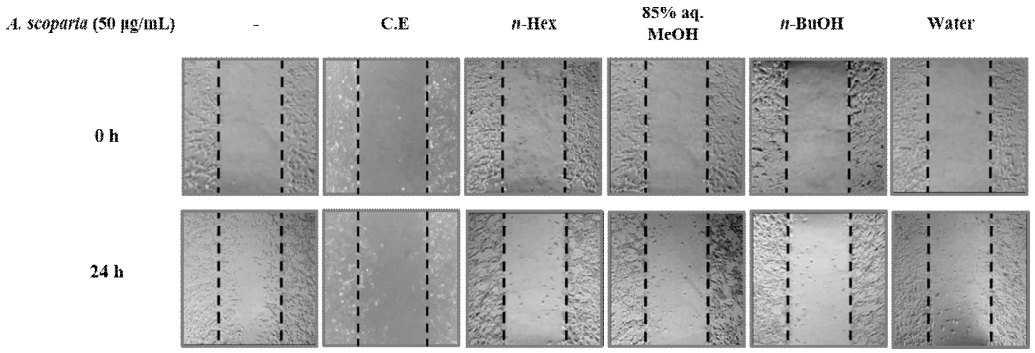


Fig. 14. Effect of crude extract and its solvent fractions from *A. scoparia* on migration ability in HT-1080 cells.

3.3.5 Effect of crude extract and its solvent fractions from *A. scoparia* on mRNA levels of MMP-9 and 2 by RT-PCR

RT-PCR was performed to confirm mRNA expression levels of MMP-9 and MMP-2 in PMA-stimulated HT-1080 cells treated with each of crude extract and its solvent fractions of *A. scoparia*. β-Actin was used as the internal control.

In cells treated with PMA alone, mRNA expression of MMP-9 and MMP-2 increased compared to cells not treated with both PMA and samples. The crude extract reduced mRNA expression levels of MMP-9 and MMP-2 to 54.9% and 52.5%, respectively (Fig. 15a).

In case of solvent fractions treatment, mRNA expression levels of MMP-9 and MMP-2 decreased except water fraction. *n*-Hexane fraction suppressed mRNA expression levels of MMP-9 and MMP-2 by 57.7% and 63.7%, showing the highest inhibitory effect, respectively. In addition, the 85% aq.MeOH fraction reduced the MMP-9 and MMP-2 mRNA expression levels to 70% and 74.1%, respectively and the *n*-BuOH fraction also reduced the MMP-9 and MMP-2 mRNA expression levels to 79% and 68.5%, respectively (Fig. 15b).

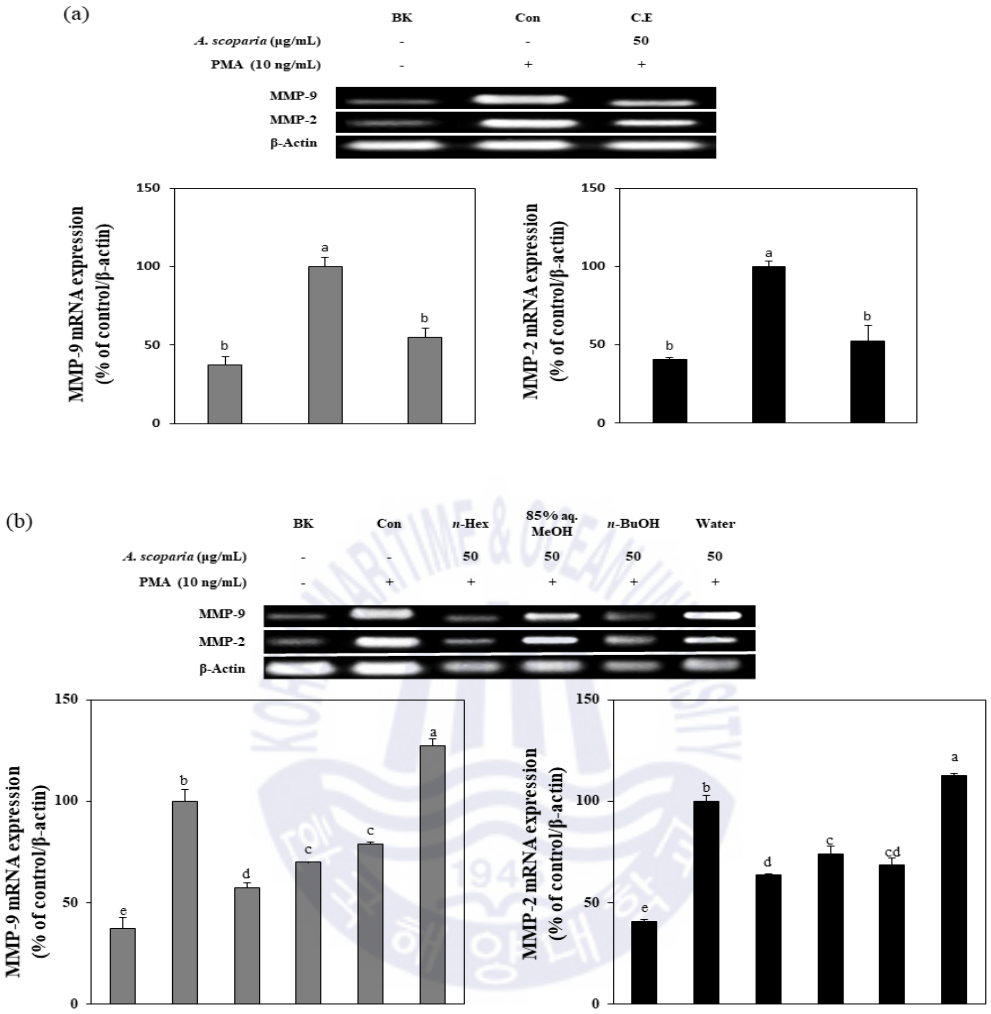


Fig. 15. Effect of crude extract (a) and its solvent fractions (b) of *A. scoparia* on mRNA levels of MMP-9 and 2 determined by RT-PCR. β-Actin was used as the internal standard. Band sizes were calculated and depicted as percentage compared to control group. Values were normalized against house keeping β-actin mRNA levels. Values are means ± SD. $^{a-e}$Means with different letters at the same concentration are significantly different ($p<0.05$) by Duncan's multiple range test.

3.3.6 Effect of crude extract and its solvent fractions on protein levels of MMP-9 and 2 by Western blot assay

Western blot assay was performed to confirm the protein expression levels of MMP-9 and MMP-2 in PMA-stimulated HT-1080 cells treated with each of crude extracts and its solvent fractions. In cells treated with PMA alone, protein expression of MMP-9 and MMP-2 increased compared to the blank. The crude extract reduced protein expression levels of MMP-9 and MMP-2 to 35.4% and 23.9% in PMA-stimulated HT-1080 cells, compared to the control, respectively (Fig. 16a).

Protein expression levels of MMP-9 and MMP-2 also decreased by treatment of the solvent fractions. Especially, *n*-hexane and 85% aq.MeOH fractions were most effective in reducing protein expression of MMP-9 and MMP-2: *n*-hexane fraction, 43.4% for MMP-9, 60.2% for MMP-2; 85% aq.MeOH fraction, 42.2% for MMP-9, 60.2% for MMP-2 (Fig. 16b).

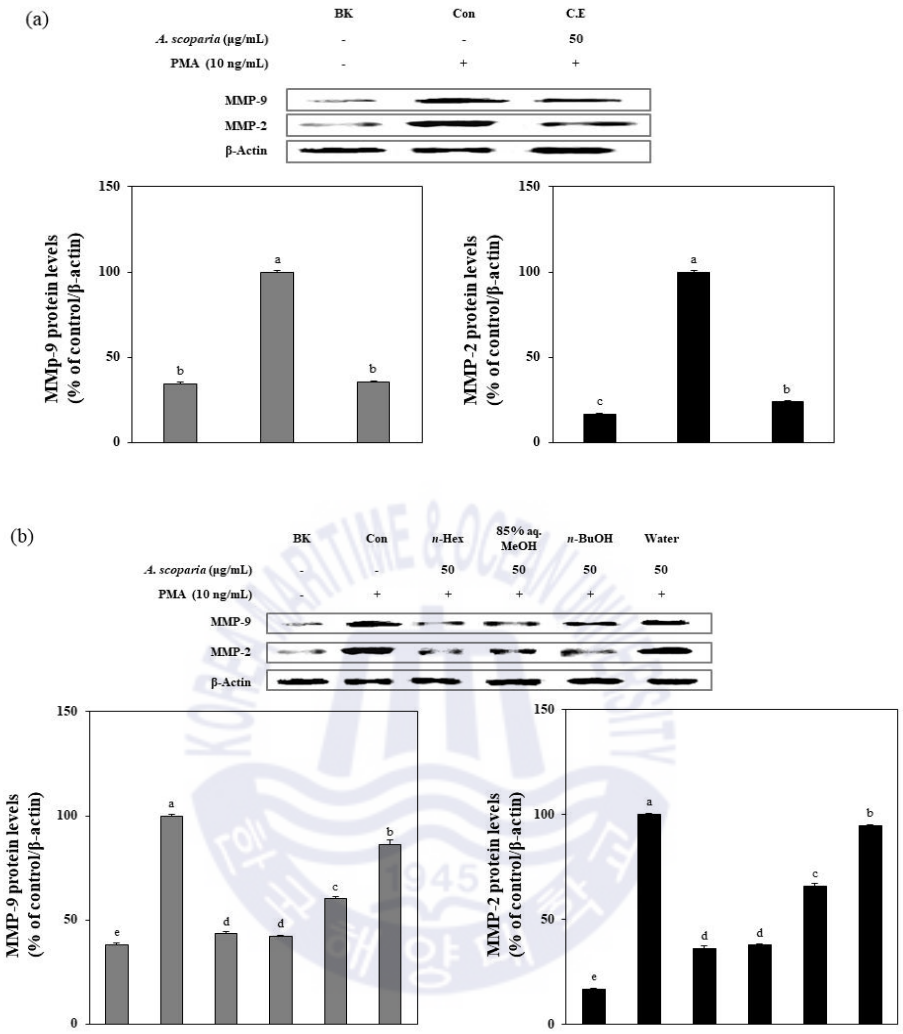


Fig. 16. Effect of crude extract (a) and its solvent fractions (b) from *A. scoparia* on protein levels of MMP-9 and 2 determined by Western blot assay. β-Actin was used as an internal standard. Band sizes were calculated and depicted as percentage compared to control group. Values were normalized against house keeping β-actin protein levels. Values are means ± SD. $^{a-e}$Means with different letters at the same concentration are significantly different ($p<0.05$) by Duncan's multiple range test.

4. Conclusion

Obesity-related diseases and cancers are on the rise worldwide, and The resulting mortality rate in Korea is a high proportion of all deaths. Obesity can be caused by several factors - mental tension, lack of physical exercise, irregular eating habits inescapable in modern society, etc. In addition, chronic inflammation due to excessive accumulation of fat can affect organs closely related with cancer, eventually leading into cancer. These cancer or tumor cells also metastasize to other organs, which can be fatal to patients. Matrix metalloproteinases, enzymes involved in invasion, angiogenesis, and metastasis of cancer cell by breaking down extracellular matrix. Therefore, much attention has been focused on MMP inhibitors to treat cancer. In particular, since synthetic MMP inhibitors have many problems in the selectivity of attack on cancer cells, efforts are being made to find MMP inhibitors from natural products (Kim et al., 2006; Kong et al., 2008; Kim et al., 2018). In this study, we searched for a substance that inhibits adipogenesis and MMPs activity from the halophyte *Artemisia scoparia*, a native plant growing on the coastal area of South Korea.

As already mentioned earlier, *A. scoparia* has been used as a folk medicine from ancient times. This plant was collected from the coast of Donggumdo, Ganghwa-gun, Incheon. It was extracted with CH_2Cl_2 and MeOH. Combined crude extracts were fractionated into *n*-hexane, 85% aq.MeOH, *n*-BuOH and water fractions by solvent polarity. The following bioassay methods were used to evaluate the inhibitory effects of *A. scoparia* on adipogenesis and MMPs activity: Oil Red O staining, RT-PCR, and Western blot assay for antiadipogenesis; gelatin zymography, ELISA, wound healing assay, RT-PCR, and Western blot assay for MMP activity.

In Oil Red O staining assay for adipogenesis, 85% aq.MeOH fraction of all

the samples most strongly inhibited formation of lipid droplets in 3T3-L1 adipocytes, and it was almost similar to the control not treated with sample. In RT-PCR, which measures expression level of obesity-related genes, *n*-hexane fraction was the most effective at inhibiting mRNA expression of SREBP1C. On the other hand, 85% aq.MeOH fraction was most effective in inhibiting mRNA expression of PPAR γ and C/EBP α. In Western blot assay, which measures protein expression level of obesity-related factors, 85% aq.MeOH fraction was most effective in reducing protein expression levels of all three obesity-related factors SREBP1C, PPAR γ and C/EBP α.

In gelatin zymography for measurement of MMP activity, it was confirmed that crude extract significantly reduced enzymatic activity of MMP-9 and MMP-2. Among solvent fractions, *n*-hexane fraction inhibited enzymatic activity most effectively. In addition, 85% aq.MeOH fraction also showed an enzymatic activity inhibitory effect close to the blank. In ELISA, among the solvent fractions, *n*-hexane and 85% aq.MeOH fractions showed significant effects on inhibition of MMP-9 and MMP-2 secretion. In case of MMP-2, the secretion was also inhibited by *n*-BuOH and water fractions. Cell migration related to cancer metastasis and invasion was observed by wound healing assay. The untreated control group showed more migration tendency than the cells treated with the samples. All samples including crude extract were effective in inhibiting cell migration. In RT-PCR, mRNA expressions of MMP-9 and MMP-2 were effectively inhibited by all solvent fractions except water fraction. In Western blot assay, protein expressions of MMP-9 and MMP-2 were most potently inhibited by *n*-hexane and 85% aq.MeOH fractions. The *n*-BuOH fraction significantly suppressed protein expressions of MMP-9 and MMP-2.

By bioactivity-guided separation, two compounds were isolated from 85% aq.MeOH fraction which showed the most potent inhibitory effect both on adipogenesis and MMP enzymatic activity. Their chemical structures were

determined to be known sesquiterpenes, reynosin (**1**) and santamarine (**2**) by the extensive 2D NMR experiments. Reynosin and santamarine have been previously isolated from several plants (El-Felary & Chan, 1978; Ito et al., 1984; Adegawa et al., 1987; Fang et al., 2005; Barla et al., 2007; Rosselli et al., 2012; Al-Attas et al., 2015), but were first isolated from the genus *artemisia*. Reynosin and santamarine have been reported to have anti-inflammatory effects by inhibiting TNF-α production in LPS-stimulated RAW 264.7 cells. In addition, these compounds were effective in inhibiting alcohol absorption (Cho et al., 1998; Yoshikawa et al., 2000).

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Appendix

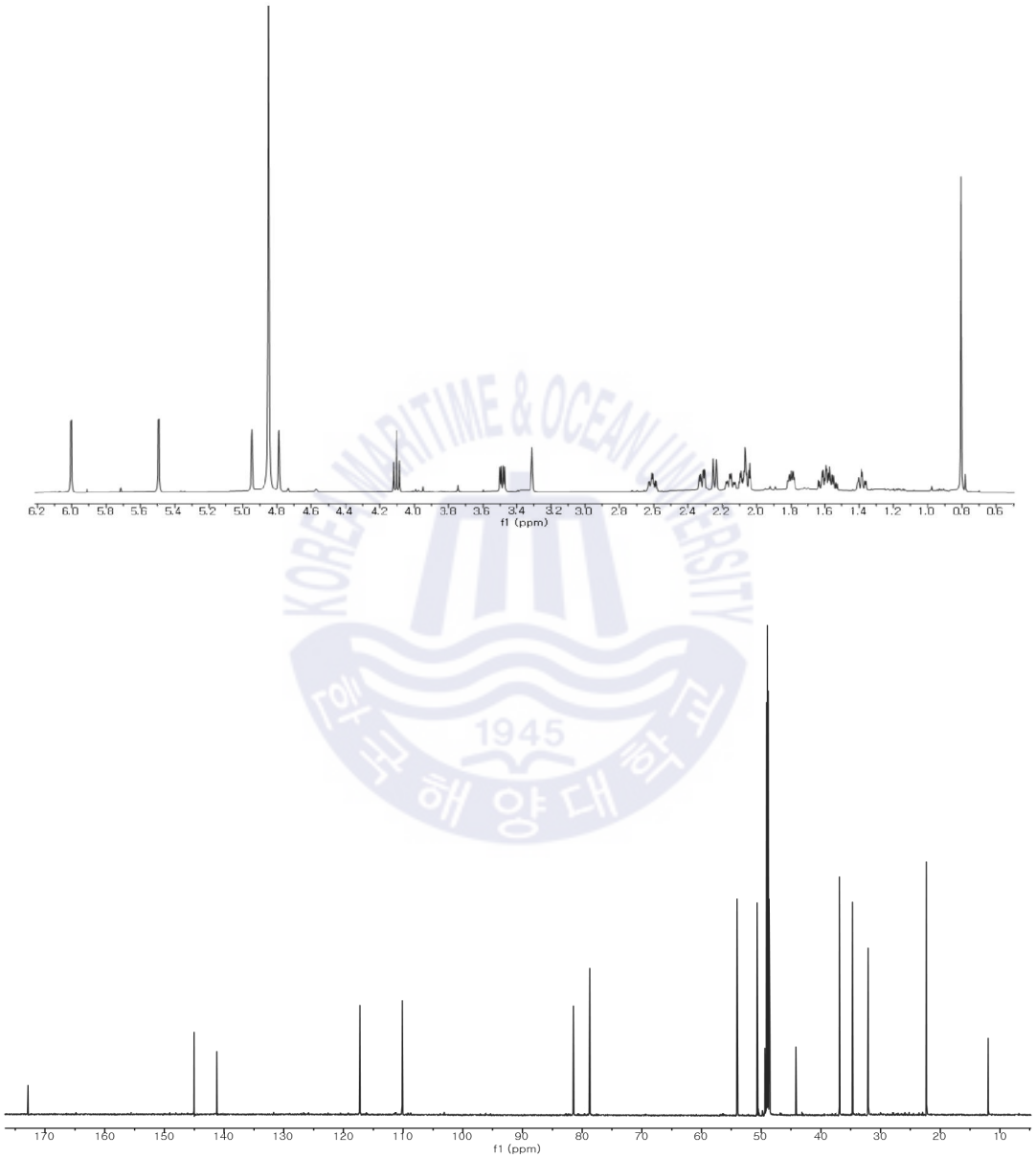


Fig. 17. ^{1}H and ^{13}C NMR spectra of compound **1** isolated from the *A. scoparia*.

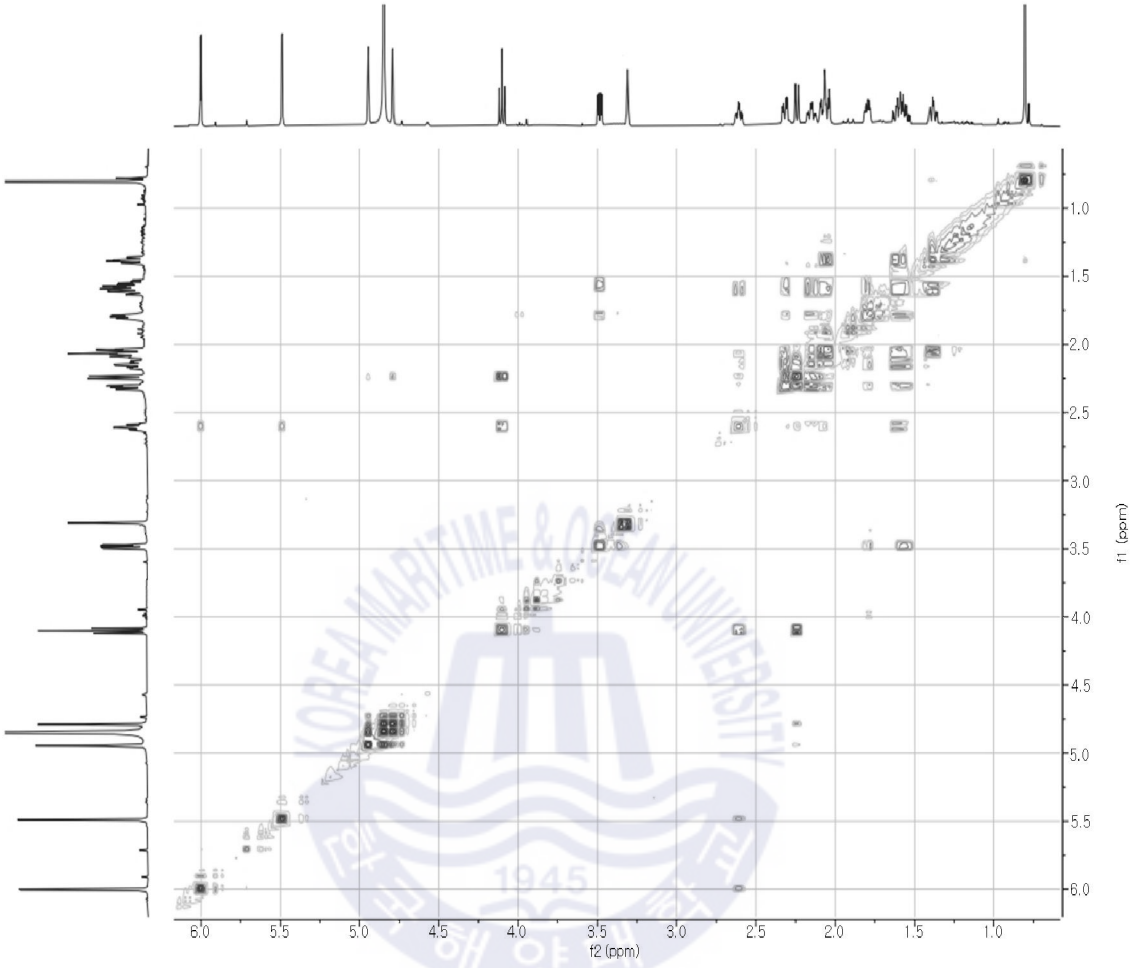


Fig. 18. ^{1}H-^{1}H COSY spectrum of compound **1** isolated from the *A. scoparia*.

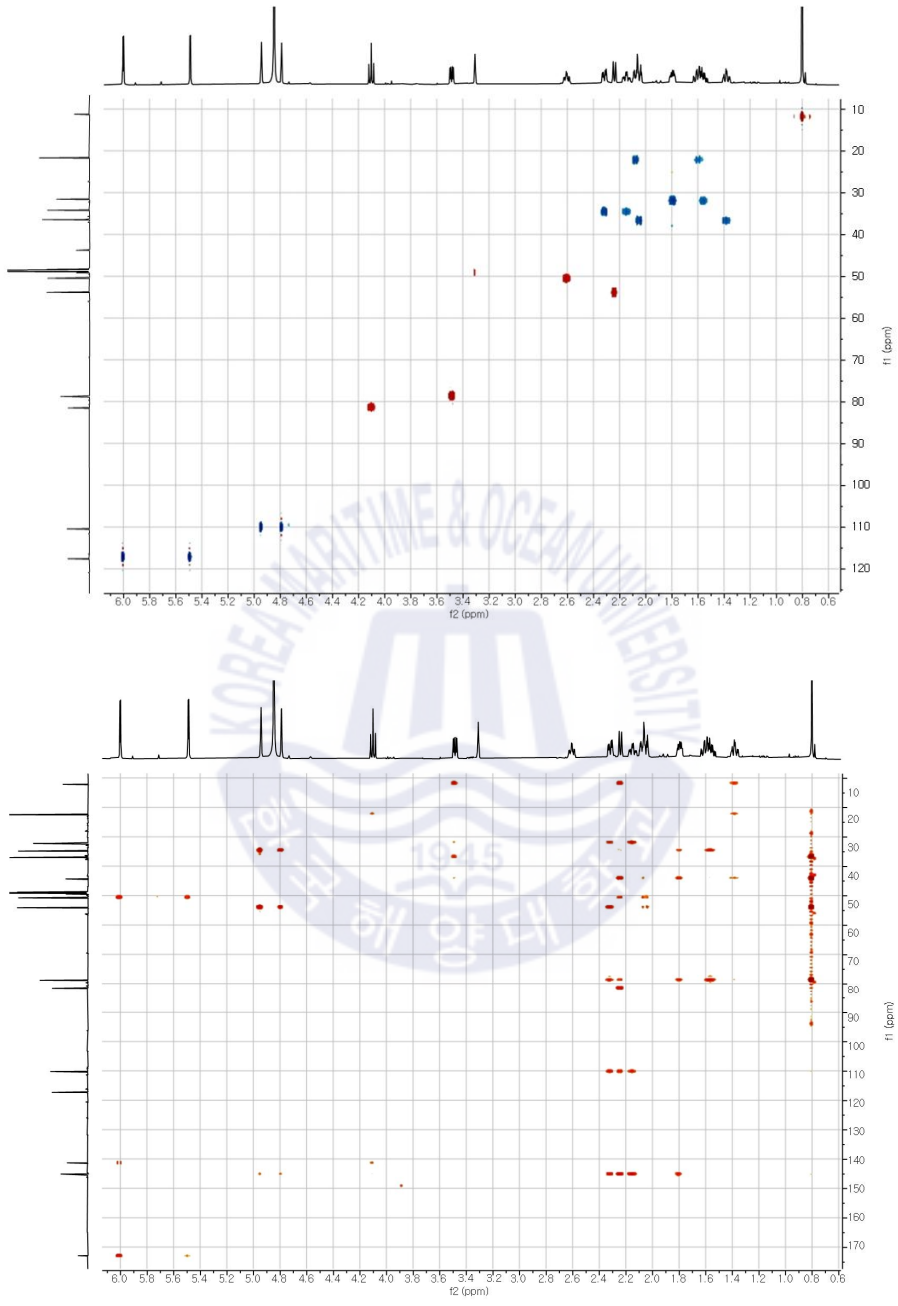


Fig. 19. gHSQC and gHMBC spectra of compound **1** isolated from the *A. scoparia*.

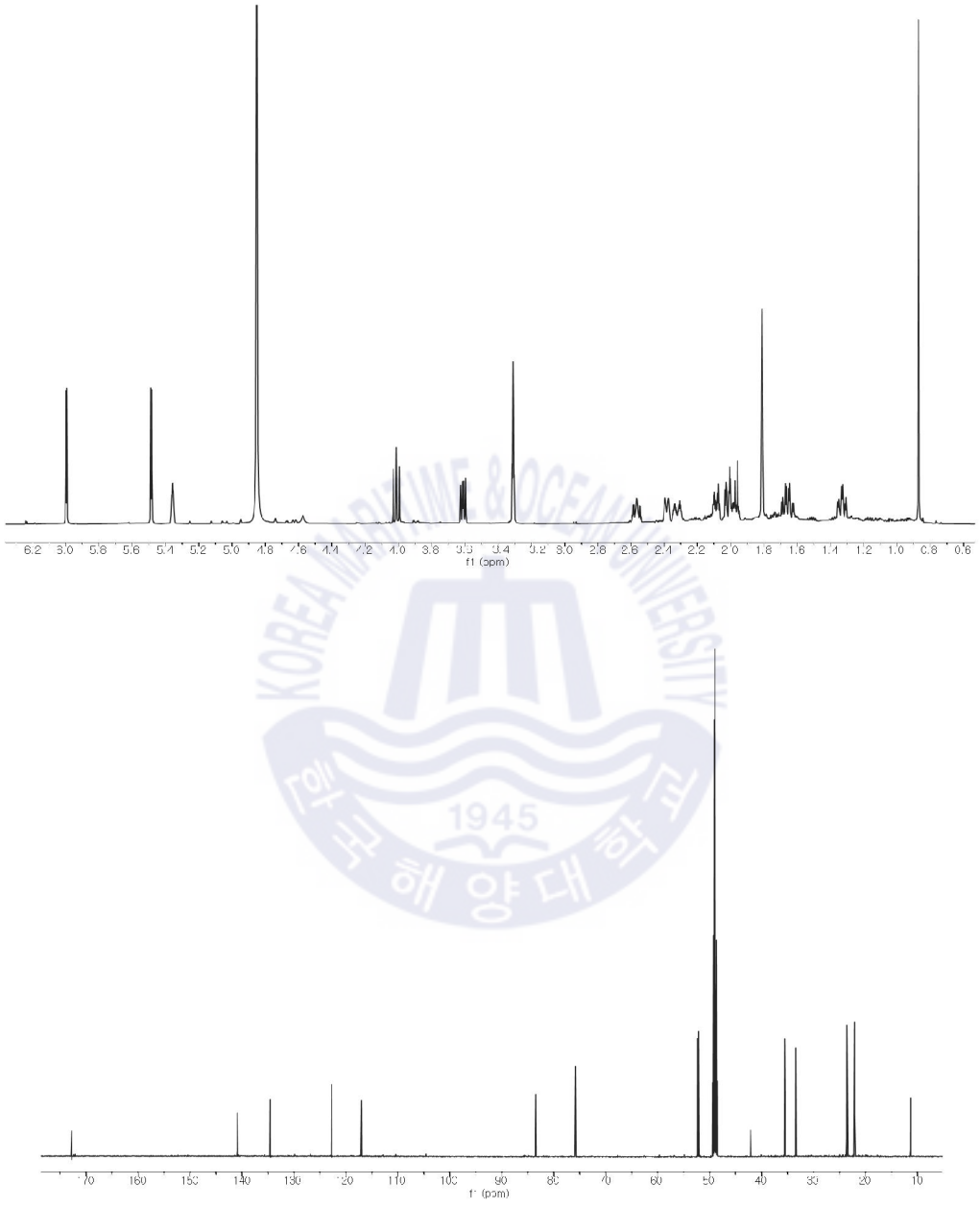


Fig. 20. ^{1}H and ^{13}C NMR spectra of compound **2** isolated from the *A. scoparia*.

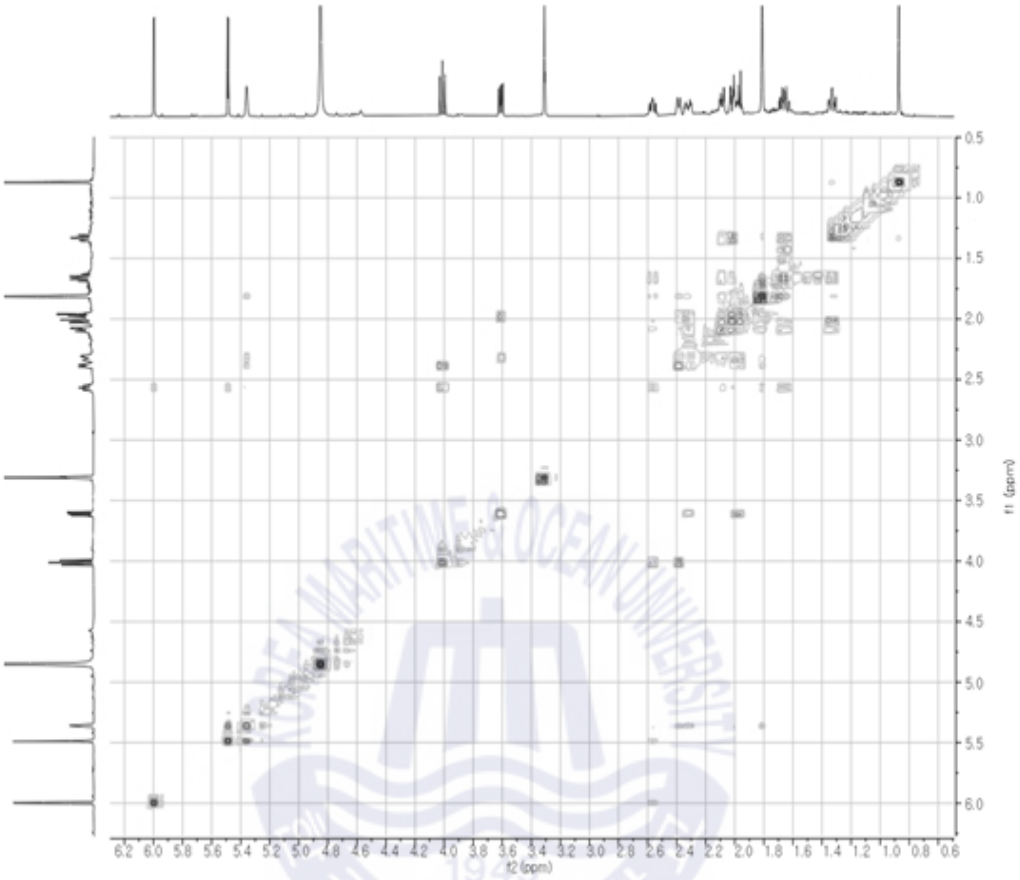


Fig. 21. ^{1}H-^{1}H COSY spectrum of compound **2** isolated from the *A. scoparia*.

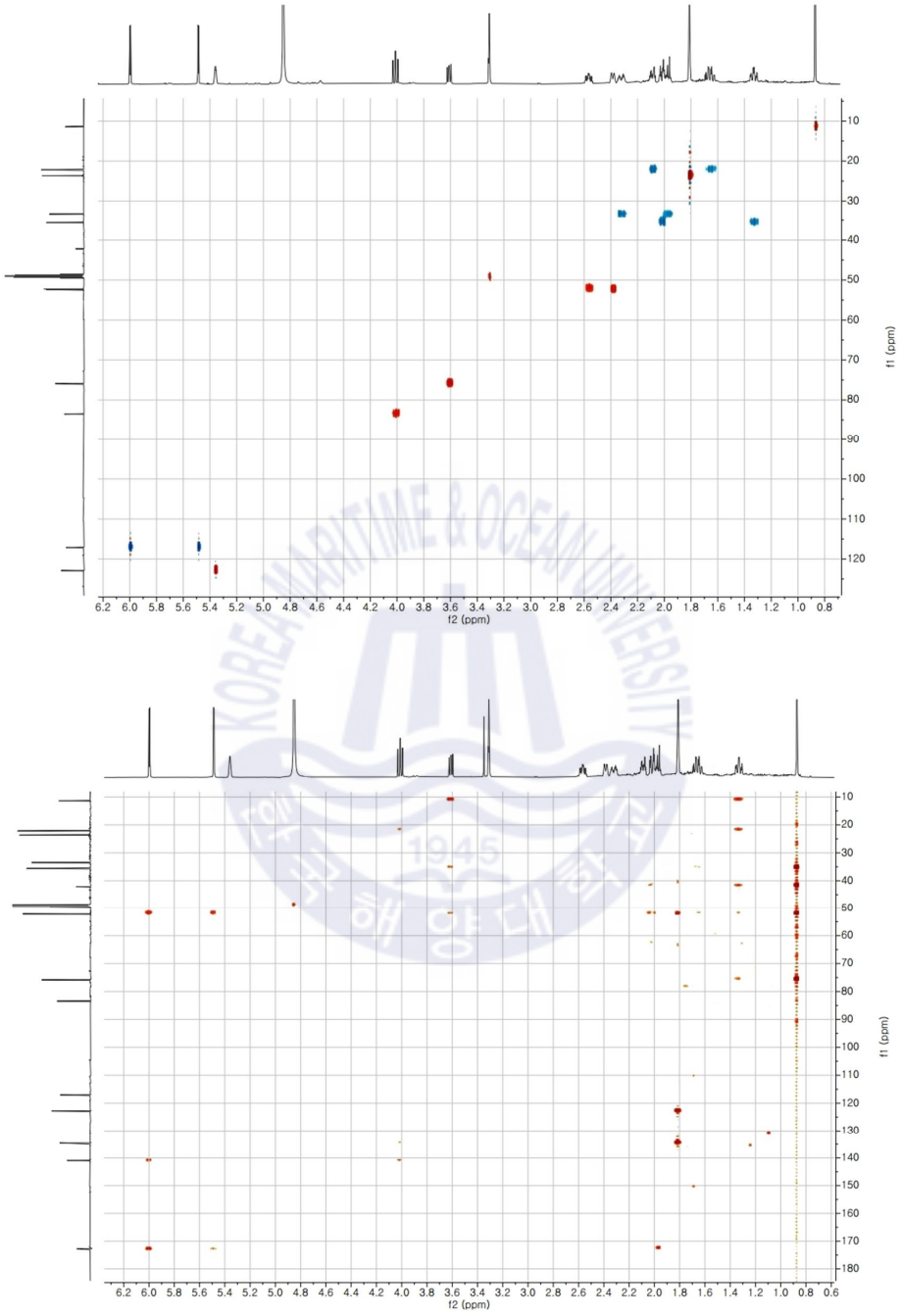
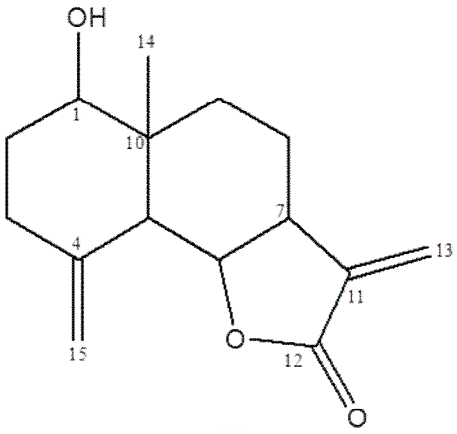
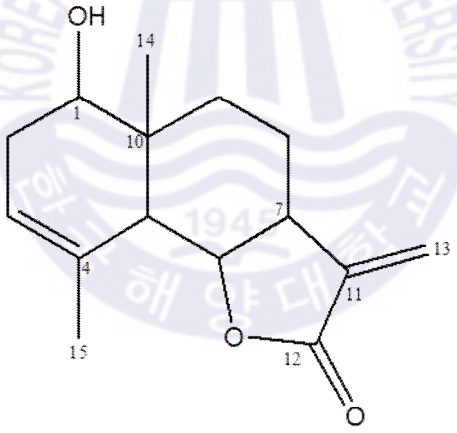


Fig. 22. gHSQC and gHMBC spectra of compound 2 isolated from the *A. scoparia*.



Compound 1



Compound 2

Fig. 23. Chemical structure of compounds **1** and **2** from *A. scoparia*.