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Screening of sirtuins (SIRTs) activators from natural products and characterization of its constituents from *Polygonum cuspidatum*

Chosun University Graduate School

Department of Pharmacy

Kim Ja Yeon

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천연물로부터 sirtuins 단백질 활성작용 탐색 및 호장근으로부터 분리한 sirtuins 활성화 물질

2012년 08월 24일

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이 논문을 약학 석사학위신청 논문으로 제출함

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Chosun University Graduate School

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List of Abbreviations

HPLC: high performance liquid chromatography

EI-MS: electro impact mass spectroscopy

IR: infrared absorption

m/z: mass to charge ratio

NMR: nuclear magnetic resonance

RP: reverse phase

SIRT1: silent information regulator two ortholog 1

UV: ultraviolet absorption

DMSO: dimethyl sulfoxide

Res: resveratrol

(국문 초록)

천연물로부터 sirtuins 단백질 활성작용 탐색 및 호장근으로부터 분리한 sirtuins 활성화 물질

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Silent information regulator two ortholog 1 (SIRT1)은 NAD 의존적 class Ⅲ histones 활성을 지닌 포유동물 상동체의 Sir2와 가장 밀접하게 관계된 핵 단백질이다. SIRT1은 신진대사 증후군, 비만, Pkinson’s 질병, 세포노화, 환경스트레스, 제2형 당뇨병과 관련된 기능이 보고되었으며, 생물학적 체계에서 다양한 기능을 형성한다. 그러므로 SIRT1 activator는 노화, 당뇨, 비만 등을 억제하는데 밀접한 관계가 있다. 최근 몇몇 저분자 물질들이 SIRT1 activator로 알려져 있지만 새로운 activator를 찾기 위한 연구는 계속되고 있다. 새로운 SIRT1 activator는 SIRT1의 생물학적 기능에 대한 이해를 개선하는데 필요하며 SIRT1의 다양한 활성은 의학적, 산업적인 가치를 기대할 수 있다.

이번 연구의 목적은 천연물로부터 SIRT1 activator를 발굴하는 것이며, 본 연구실에서 수행한 sirt1-p53 luciferase assay 통하여 약 천 여개의 한국약용식물의 SIRT1에 대한 활성을 측정한 결과 여러 후보 물질 중 *polygonum cuspidatum* MeOH 추출물에서 SIRT1에 대한 강한 활성을 나타내는 것을 확인하였다. 활성화시

키는 화합물은 anthraquinone계 골격을 갖는 화합물로 Physcion, Aloe-emodin, Emodin, 2-methoxystypandrone, Resveratrol 이며, 분리된 화합물들의 구조는 각종 스펙트럼 방법 (1D, 2D-NMR, UV, IR, MS)과 보고된 문헌을 참고하여 동정하였다. 분리한 화합물 **1 - 5**의 *in vitro* SIRT1 효소에 대한 활성을 측정한 결과, 화합물 **1 - 5**는 SIRT1 대하여 강하게 activation 시키는 것을 확인하였다. 화합물 **1 - 5**에 의해서 p53의 transcription activity가 강하게 감소하였으며, SIRT1 deacetylation activity와, NAD-NADH ratio 가 증가하는 것을 확인하였다. 뿐만 아니라 화합물 **4**는 노화 세포인 HDF에 처리시 젊은 세포 형태로 cell morphology의 변화와 노화지표인 β-galactosidase 활성이 감소하는 것을 최종적으로 확인할 수 있었다.

1. Introduction

1.1. SIRT1 enzyme

Sirt1 (mammalian) belongs to a member of the sirtuin family [1]. It has a role of a nicotinamide adenosine dinucleotide (NAD) – dependent deacetylase and also can eliminate acetyl groups from many histone, nonhistone proteins [2]. Therefore many substrates can be deacetylated by Sirt1. Sirt1 involve in a wide range of physiological functions such as it controls apoptosis/cell survival, differentiation, metabolism, gene expression and aging.

Sirt1 and its ortholog play a major role in determining lifespan of yeast, files and mice [3-5]. In human stem cells, aging is related to telomere erosion. In the absence of telomerase, telomere becomes short and then it causes a change in structure of chromatin. This is exhibited by the DNA-damage response pathway and activates programmed cell death. Sirt1 is able to be an important regulator of telomere maintenance during stem cell aging because it has essential role in gene silencing [6]. Sirt1 could influence stem cell aging and ultimately contribute to longer lifespan so it is also a potential mechanism in maintenance of telomere stability via histone deacetylation and chromatin stability modification [7]. Furthermore, many studies demonstrate that caloric restriction (CR) can delay the aging process and retard the onset of numerous aging-related disease.

As another way Sirt1 could be concerned with stem cell aging via its vital role in caloric restriction (CR) – induced lifespan extension [8-9] and it is associated to age-related reactive oxygen species (ROS) generation [10-12].

1.2. SIRT1 function

In mammals, sirtuins are categorized into seven different classes (Sirt1-7). Sirt1 was reported that it was involved in age related disorders such as type II Ⅱ diabetes mellitus, obesity, metabolic

syndrome, and Parkinson's disease. Sirt1 activating is one of the promising approaches to give medical treatment to these age related disease. In several adult tissues, sirtuins are largely expressed such as brain, heart and skeletal muscle. Sirt1 is known to interact with a number of influential transcription factors like p53, p65 (NF-kB), MyoD and modulates their activities [13]. Therefore, sirtuins are important targets to design activators and inhibitors. In this work, we mainly focus on Sirt1.

Sirt1 is an essential regulator of pathways downstream of caloric restriction that produce helpful effects on glucose homeostasis and insulin sensitivity [4, 8, 14-18]. Recent studies illustrated that activators of Sirt1 improve whole-body glucose homeostasis and insulin sensitivity in adipose tissue, skeletal muscle and liver. Thus, activation of Sirt1 is a hopeful new therapeutic approach for treating diseases of aging like type Ⅱ diabetes

1.3. SIRT1 activators

Some activators have been described, e.g., resveratrol, fisetin and butein, however all of these compounds are plant polyphenols [19-20]. Resveratrol is a nature substrate, which can be found in red wine, activates Sirt1 and increases lifespan of mammals [21-22]. It has the capability to scavenge oxidatively generated free radicals [23-30], and it reveals cancer preventative properties [31-35]. Resveratrol was shown to improve Sirt1-dependenct cellular processes like axonal protection [36], fat mobilization [37], and inhibition of NF-kB – dependent transcription [38].

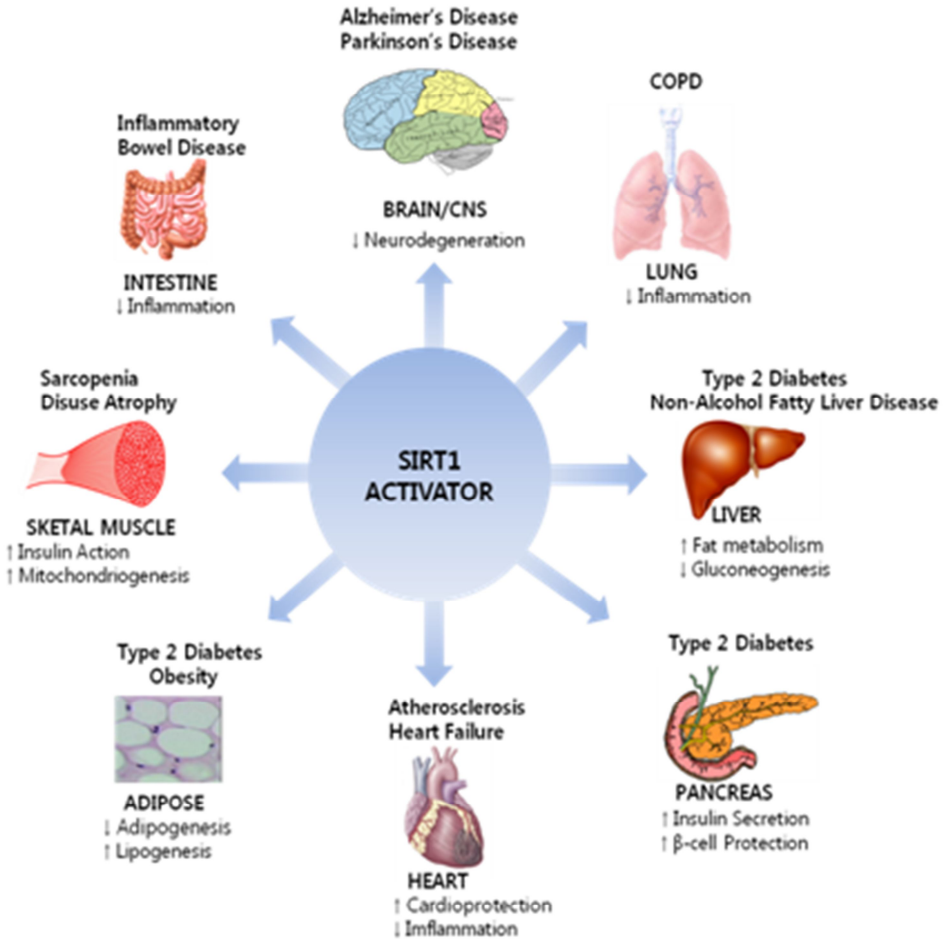


Figure 1 : Activating SIRT1: Broad potential for diseases of aging

1.4. Screening of the Korea medicinal plant through luciferase assay

We screened one thousand Korean medicinal plants' extracts library using SIRT1-p53 luciferase assay to find SIRT1 activator. Luciferase assay is really high throughput method. This method uses firefly luciferase gene (luc) expression produces the enzyme luciferase which converts the substrate D-luciferin to non-reactive oxyluciferin, resulting in green light emission at 562nm. After three plant's extracts were combined to make a group (TCMU, TCU), these mixture units were screened for extract groups that exhibited potentially stimulation on SIRT1 activity. And then, we screened for after divided as single plant extract of candidate mixture groups with the activation effect on SIRT1. From these screening procedures, we can get the candidates with potential activation of SIRT1.

1.5 *Polygonum cuspidatum*

Poligoni cuspidati is the dried rhizome (stem & root) of *Polygonum cuspidatum* Sieb. et Zucc. belongs to Polygonaceae family. It is called Hu-Zhang in Chinese and Korea and Ko-Jo-Kon in Japanese.

Figure 2: The roots, stems, leaves, and flowers of *Poligonum cuspidatum*

The use of this plant in folk medicine has long been applied for the treatment of atherosclerosis, hypertension, cough, suppurative dermatitis and gonorrhoea, hyperlipidemia as well in Chinese, Korea and Japan. *Polygonum cuspidatum is a large,* herbaceous perennial plant, native to eastern Asia in Japan, China and Korea. It is an invasive flowering plant used to produce resveratrol supplements because it is easy and quick to grow. It has become popular since it is a rich source of resveratrol. Emodin and physcion are also abundant anthraquinone compounds found in this traditional medicinal plant. An extract of *Polygonum cuspidatum* can be taken as a dietary supplement that is usually made from the roots of the plant.

Previous chemical studied of *Poligonum cuspidatum* (PC) have shown the presence of anthraquinones such as emodin, stilbenes (resveratrol) and other phenolics. The anthraquinone derivatives isolated from this plant are known to have a variety of biological activities, such as cell adhesion inhibitory, anti-inflammatory, murine neuroleptic, hepatoprotective effect, antifungal, antiviral and anticancer activities. It has also been used for the treatment of atherosclerosis, platelet aggregation, apoptosis, blood cholesterol, and diabetic nephropathy.

2. Materials and Methods

2.1. Materials

2.1.1. Plant

The dried roots of *Poligononum cuspidatum* were purchased at a medical market of Gwangju city, in Republic of Korea. The sample was identified by professor Oh, W. K. and its voucher specimen (No. 2011-0010) has been deposited at the Department of Pharmacy, Chosun University, Republic of Korea.

2.1.2. Chemicals, reagents and chromatography.

Column chromatography was conducted on silica gel (Merck, 63 – 200 μm particle size) and reversed phase (ODS-A, Merck, 120 μm particle size) from Merck. TLC was carried out with silica gel 60 F254 plates from Merck. The solvent for NMR analysis was purchased from CIL (Cambridge Isotope Lab., USA). HPLC solvents were from Burdick & Jackson, USA.

Resveratrol were purchased from Sigma Chemical Company (St Louis, MO, USA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from GIBCO-BRL (Grand Island, NY, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole)] was purchased from USB Corporation (Cleveland, Ohio, USA). Human recombiniant SIRT1, Fluor de Lys SIRT1 deacetylse substrate, Fluor de Lys Developer II, NAD^+ and the buffer used for assays were purchased from Biomol (Plymouth Meeting, PA), and NAD/NADH Quantitation kit was purchased from Biovision

2.1.3. General experimental procedures.

UV spectra were taken in MeOH using a Shimadzu spectrometer. The nuclear magnetic resonance (NMR) spectra were obtained on a FT-JEOL 300 MHz spectrometer at Chosun University and a Varian Unity Inova 500 MHz spectrometer at Korea Basic Science Institute (KBSI, Gwangju Center, Korea). EI-MS data were performed on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. HPLC was carried out using a Gilson System with UV detector and an RP-C18 column (10 × 250 mm, 10 μm particle size, RS Tech, Korea).

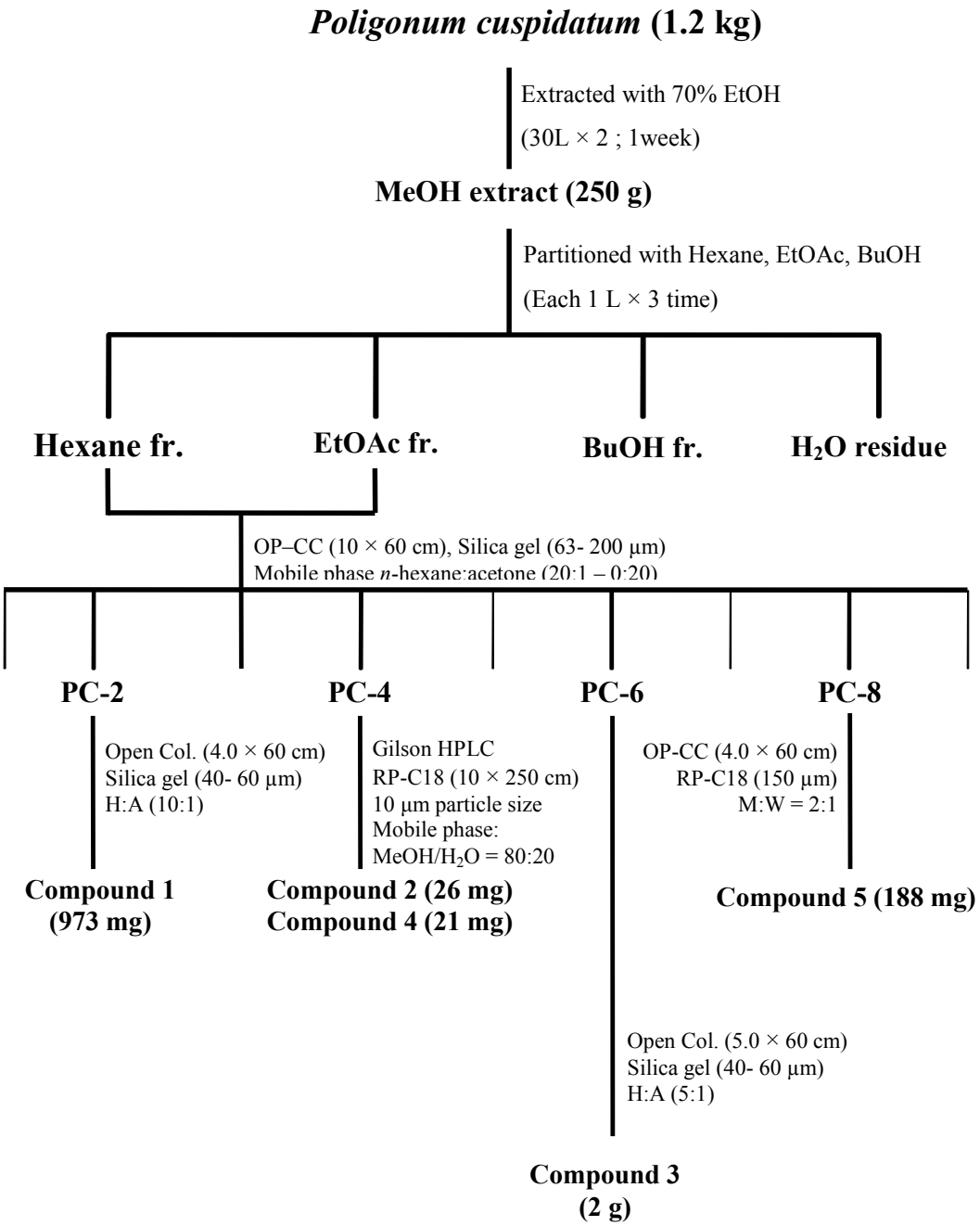
2.2. Methods

2.2.1. Extraction and isolation of active compounds on SIRTs from *Poligonum cuspidatum*

The dried roots of *Poligonum cuspidatum* (PC, 1.2 kg) were extracted with MeOH at room temperature using sonication for 1 day. Then the methanol-soluble extract was filtered and concentrated under reducing pressure to give a brown extract of 250 g. This was dissolved in 1L of water and then successively partitioned with n-hexane, EtOAc, and n-BuOH (each 1 L × 3 time), gave hexane fraction (Hx fr. 20 g), EtOAc fraction (EA fr. 100 g), and BuOH fraction (Bu fr. 70 g), respectively.

When these fractions were checked on Sirt1 assay, it revealed that the Hx fr. and EA fr. were most active fraction. A part of this combined fraction (65 g) was further chromatographed over a silica gel column (10 × 60 cm; 63 – 200 μm particle size) using a gradient of *n*-hexane/acetone (from 20:1 to 1:20), to yield ten fractions (PC.1 – PC.10) according to their TLC profiles. Compound **1** (973 mg) was isolated by rechromatography of fraction 2 (PC-2) on a silica gel column (4 × 60 cm; 40 – 60 μm particle size), eluted with an isocratic solvent of 90 % of hexane in acetone. Compound **3** (2 g) was purified from a part of fraction 6 (PC-6) by crystallization with 30%

EtOAc in *n*-hexane. Purification of fraction 4 (PC-4) by preparative Gilson HPLC systems [using OptimaPack C18, 250 × 10 mm I.D, 10 μm particle size); mobile phase MeOH and H_2O containing 0.1% formic acid (isocratic 80:20); flow rate 2 mL/min; UV-detections at 205 and 254 nm] resulted in the isolation of compound **4** (21 mg, t_R 21.5 min) and compound **2** (26 mg, t_R 28 min), respectively. Fraction 8 (PC-8) was applied to column chromatography over a RP-C18 column (4 × 40 cm, 150 μm particle size), eluted with 65 % MeOH in H_2O, resulted in the isolation of compound **5** (188 mg), respectively.



Scheme 1. Isolation scheme of compounds **1**–**5** isolated from the *P. cuspidatum*

Physcion (1):

Yellow powder; UV λ_{max} (MeOH) nm: 300, 253; IR υ_{max} (KBr) cm^{-1}: 3403, 2916, 1676, 1664, 1418, 1433, 1321, 1240, 1184, 1155-756; EI-MS m/z (rel int) 284 $[M]^+$; ^1H and ^{13}C NMR data, see Table 1.

Aloe-emodin (2):

Yellow powder; UV (MeOH) λ_{max} nm: 280, 235; IR υ_{max} (KBr) cm^{-1}: 3390, 2959, 1591, 1515, 1417, 1159-1028; EI-MS m/z (rel. int.): 270 $[M]^+$; ^1H and ^{13}C NMR data, see Table 1.

Emodin (3):

Yellow powder; UV (MeOH) λ_{max} nm: 254, 273, IR υ_{max} (KBr) cm^{-1}: 3400, 2957, 2924, 1634, 1506, 1457, 1435, 1091,1031. EI-MS m/z (rel. int.): 270 $[M]^+$; ^1H and ^{13}C NMRdata, see Table 1.

2-methoxystypandrone (4):

Yellowish powder; UV λ_{max} (MeOH) nm: 208.5, 235.6, 286.2; IR υ_{max} (KBr) cm^{-1}: 3490, 2960, 2920, 2900, 1605, 1595; EI-MS m/z: 260 $[M]^+$ ($C_{14}H_{12}O_5$); ^1H and ^{13}C NMR data, see Table 2.

Resveratrol (5):

White amorphour powder; UV (MeOH) λ_{max} nm: 210.0, 232.5, 282.5; IR (KBr) cm^{-1}: 3460, 2962, 2910, 2900, 1610, 1595; EI-MS m/z: 228.0786 ($C_{14}H_{12}O_3$); ^1H and ^{13}C NMR data, see Table 2.

2.2.2. *In vitro* SIRT1 assay

Cell culture: 293T (human embryonic kidney cells), HDF (human diploid fibroblast cells) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Welgene, Korea) supplemented with 10% fetal bovine serum and 1% Antibiotic/Antimycotic at 37^{o}C and 5% CO_2

Measurement of cell viability: Cells were seeded into 96-well plates at a density of 2 × 10^3/well. Cytotoxicity of the isolates was determined by a MTT assay. After incubation of cells with the compounds for 12 hours, cells were then treated with MTT solution for 40 minutes. The dark blue formazan crystals that formed in intact cells were solubilized with DMSO, and the absorbance at 550 nm was measured with a microplate reader (Varioskan, Thermo Electron Co.). Percent cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the control vehicle.

In vitro SIRT1 deacetylation with NAD/NADH assay: The SIRT1 enzyme reaction was performed in a final volume of 25 μL per well in a 384-well microplate. A standard SIRT1 reaction contained 0.1 U/mL enzyme, 10 μM NAD^+, 20 μM Ac-p53 (HLKSKKGQSTSRHKK(Ac)LMFK) peptide in the absence or presence of the compound in the SIRT1 assay buffer (25 mM Tris/Cl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1mM $MgCl_2$, 1mg/mL BSA). The assay buffer and the enzyme/compound mixture (10 μL) were incubated for 10 min at room temperature. The reaction was then initiated by the addition of 15 μL of the solution containing Ac-p53 peptide and NAD^+. After incubation for 6 hr at the ambient temperature, the reaction was quenched by the addition of 25 μL NAD/NADH recycling enzyme mixture containing 1 mM nicotinamide. After 2 hr incubation, the absorbance increase in the reaction was measured using VersaMax Absorbance Microplate Reader (Molecular Device, Inc.). The positive control for each plate was SIRT1 reaction in the presence of DMSO, and the negative control was reaction mixture without enzyme.

In vivo SIRT1 deacetylation with luciferase assay*:* To determine promoter activity, we used a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, cells were plated in 24-well plates at a density of 10^5 cells/ well, overnight and transiently co-transfected with p53, p53-Luc or SIRT1 and pRL-SV plasmid (Renilla luciferase expression for normalization) (Promega, Madison, WI) using LipofectAMINE™ 2000 reagent (Invitrogen, Carlsbad, CA). The cells were then exposed to the compounds for 24 hours. Luciferase activities in cell lysates were measured using a luminometer (TD-20; Turner Designs, Sunnyvale, CA). Relative luciferase activities were calculated by normalizing SIRT1 driven firefly luciferase activity to Renilla luciferase activity (Luminoskan Ascent, Thermo Electron Co.).

NAD^+-to-NADH ratio assay*:* The NAD^+-to-NADH ratio was measured from whole-cell extracts of 293T cells using the Biovision NAD/NADH Quantitation kit according to the manufacturer's instructions.

Senescence associated β–galactosidase staining assay: To prepare for assay, HDF cells (passage 38) were seeded in 12 well plates (10^5 Cells/well), after 1 days cells then would be incubated with compounds for 3-5 days. Senescent cells were washed with phosphate buffered saline (PBS) and fixed for 10 min (at room temperature) in 3% formaldehyde in PBS. After washing with PBS, cells were incubated at 37 ^oC with fresh senescence associated *β*–galactosidase staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside (X-gal), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$). Sixteen hrs later, senescent cells were identified under a light microscope.

3. Results

3.1. Structural determination of isolated compounds from *Poligonum cuspidatum*

3.1.1. Structure determination of compound 1

Compound **1** was obtained as yellow powder with the molecular formula of $C_{16}H_{12}O_5$ as determined by EI-MS. Its UV spectrum showed two absorption maxima at 253 and 300 nm. The IR (KBr) showed absorption bands for hydroxy (3403 cm^{-1}), carbonyl groups (1676, 1664 cm^{-1}) and benzene ring (1602, 1573 cm^{-1}) moieties. The ^{1}H and ^{13}C NMR spectra showed the characteristic of an anthraquinone [39] with two set of *ortho*-coupled aromatic protons δ_H 7.05 (1H, d, 1.5 Hz, H-2), 7.60 (1H, d, 1.5 Hz, H-4), and δ_H 7.33 (1H, d, 2.4 Hz, H-6), 6.65 (1H, d, 2.4 Hz, H-8), two conjugated hydroxy at δ_H 12.29 (1H, s, 9-OH) and 12.09 (1H, s, 1-OH), with two ketone carbons in the ^{13}C NMR spectrum δ_C 190.8 (C-10) and 182.0 (C-5). In addition, the ^{1}H and ^{13}C NMR displayed an aromatic benzyl (δ_H 2.43, 3H, s) and a methoxy group (δ_H 3.91, 3H, s) with corresponding carbons at δ_C 22.2 and 56.1, respectively. Therefore, compound **1** was elucidated as 1,9-dihydroxy-7-methoxy-3-methyl-9,10-anthraquinone (Physcion) [40], a major anthraquinone from *Poligonum cuspidatum*.

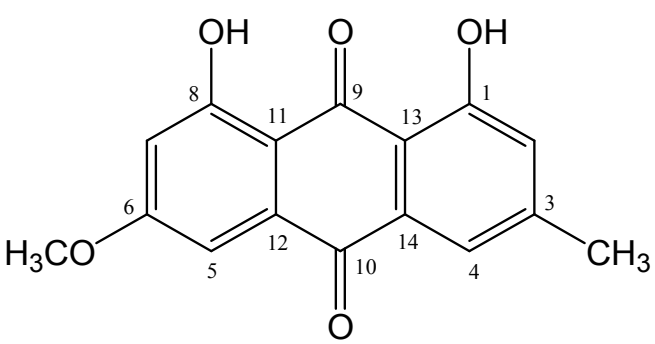


Figure 3: Chemical structure of Physcion (1)

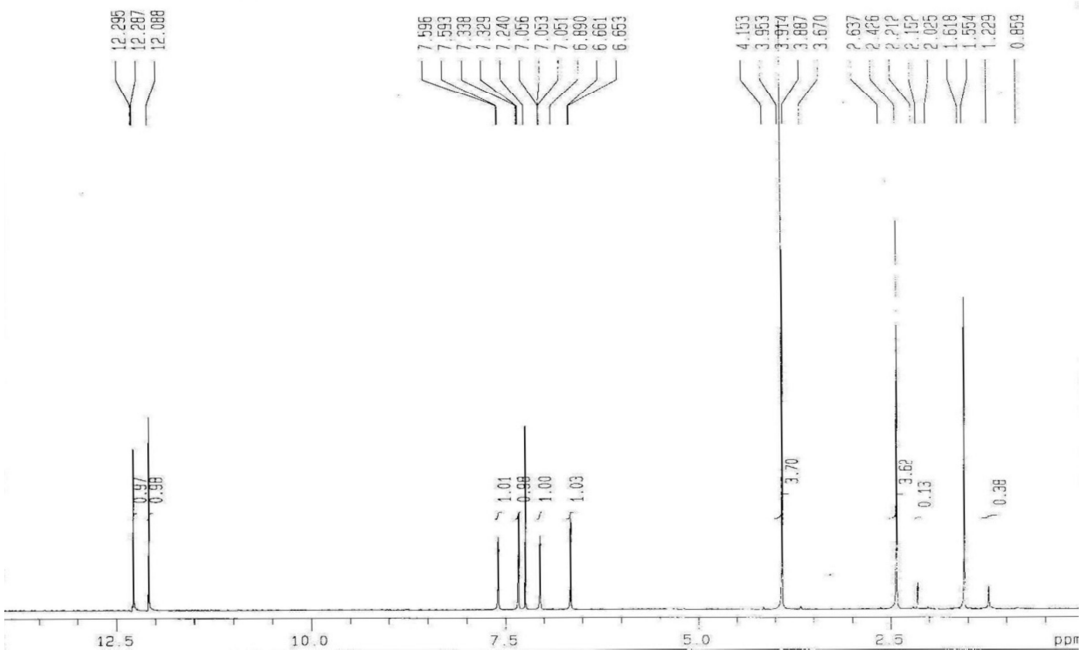


Fig. 4. ^{1}H-NMR spectrum of compound 1 (300 MHz, $CDCl_3$)

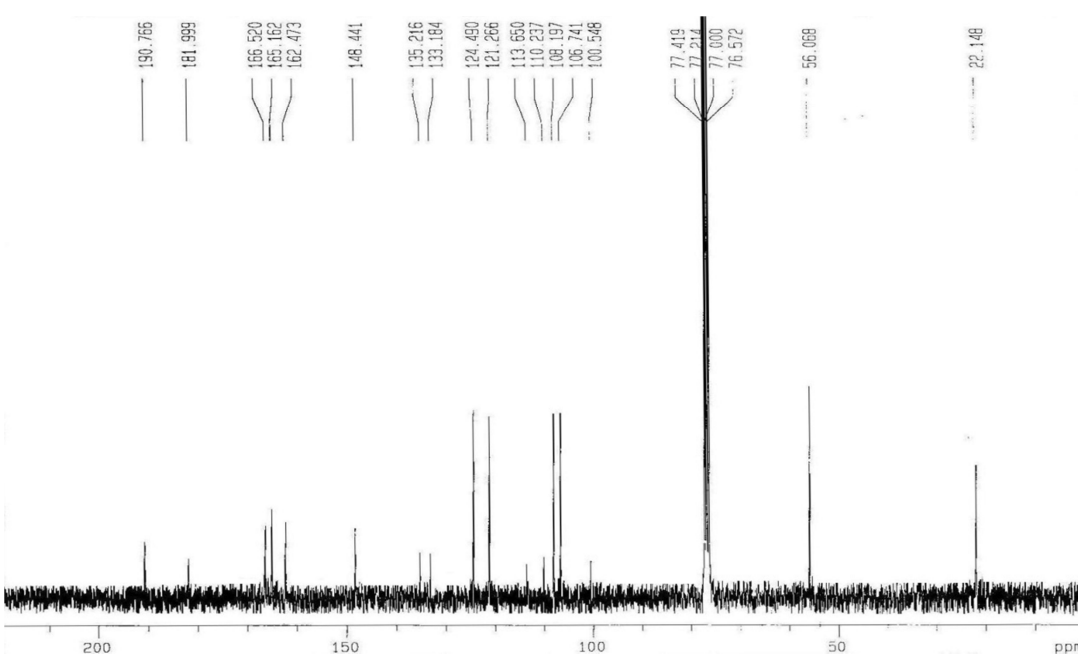


Fig. 5. ^{13}C-NMR spectrum of compound 1 (75 MHz, $CDCl_3$)

3.1.2. Structure determination of compound 2

Compound **2** was purified as yellow powder, with the molecular formula $C_{15}H_{10}O_5$. It was yellow crystal form when subjected to MeOH:$CHCl_3$ (5:95). All of the physicochemical data (UV, IR, and MS) were in good agreement with literature [41]. The ^{1}H and ^{13}C NMR spectra displayed one set of *ortho*-coupled proton signals which were assignable for H-2 (δ_H 7.26, br, s) and H-4 (δ_H 7.69, br, s), two ketone at δ_C 192.4 (C-9), 182.0 (C-10), and two oxygenated quaternary carbons at δ_C 162.5 and 162.0 for C-1 and C-8, respectively. The remaining three aromatic protons were belong to ring A [δ_H 7.74 (1H, dd, *J* = 1.5, 8.4 Hz, H-5), 7.60 (1H, br, d, *J* = 8.4, H-6), and 7.22 (1H, dd, J = 1.5, 8.4, H-7)]. The methylenehydroxy was appeared at 4.65 (2H, s) with corresponding carbon at 63.1 ppm. Thus, compound 2 was determined to be Aloe-emodin [40]. an 1,8-dihydroxyl-3-methylenehydroxyl-9,10-anthraquinone. This compound was also a major component presented in *P. cuspidatum*.

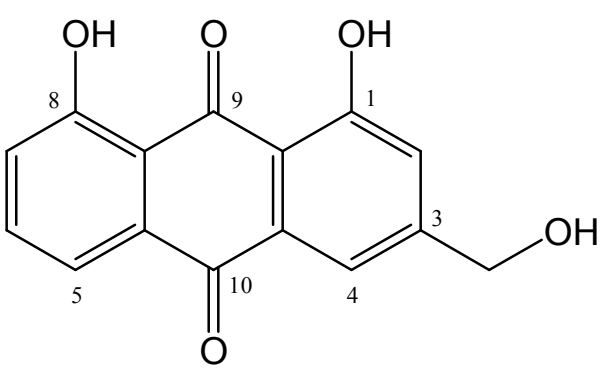


Figure 6: Chemical structure of Aloe-emodin (2)

3.1.3. Structure determination of compound 3

Compound **3** was isolated as yellow powder, with the molecular formula $C_{15}H_{10}O_5$. All of the physicochemical and the ^1H and 13C NMR spectroscopic data of **3** were very similar to that of physcion (**2**), except only for an additional methoxy moiety in **2** was disappeared in **3**. Two set of *ortho*-coupled aromatic protons at δ_H 7.05 (1H, d, 1.5 Hz) and δ_H 7.60 (1H, d, 1.5 Hz), and δ_H 7.33 (1H, d, 2.4 Hz) and δ_H 6.65 (1H, d, 2.4 Hz) were assigned as H-2, H-4, H-6 and H-8, respectively. Two conjugated hydroxy proton signals at δ_H 12.29 (1H, s) and δ_H 12.09 (1H, s) for 1-OH and 9-OH were appeared, the remaining signals at δ_H 2.43 (s, 3H) was a 3-methyl group. Therefore, compound **3** was determined to be emodin.[42]

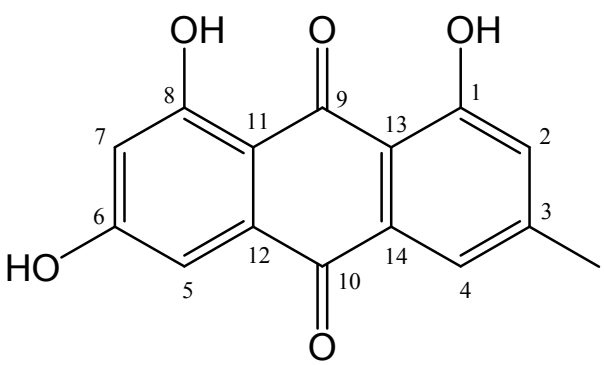


Figure 7: Chemical structure of Emodin (3)

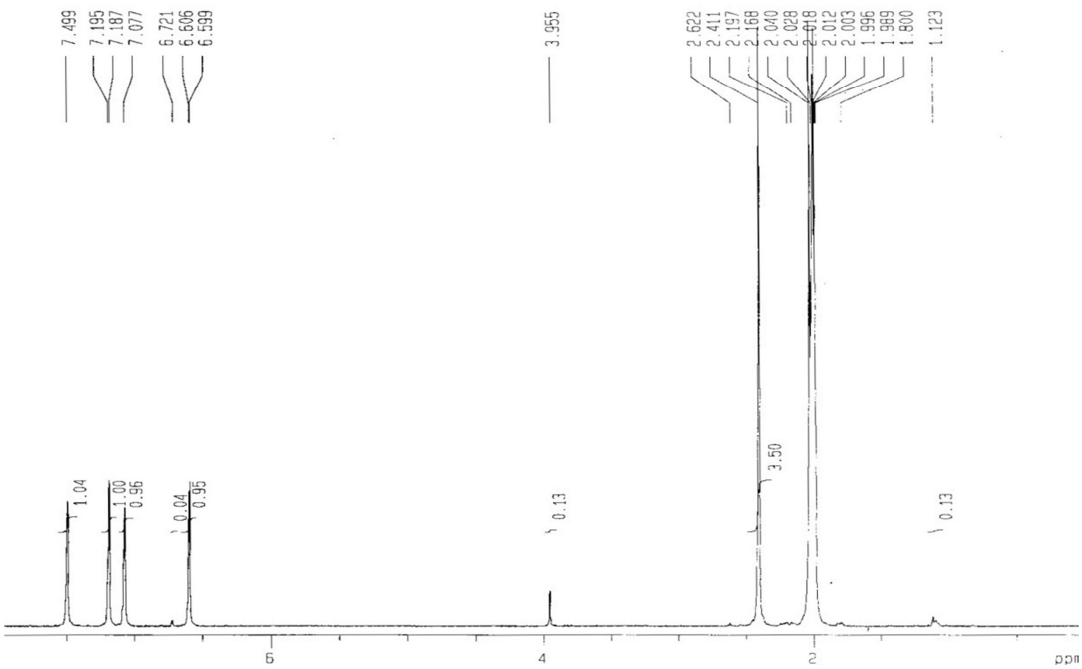


Fig. 8. ^{1}H-NMR spectrum of compound 3 (300 MHz, acetone-d_6)

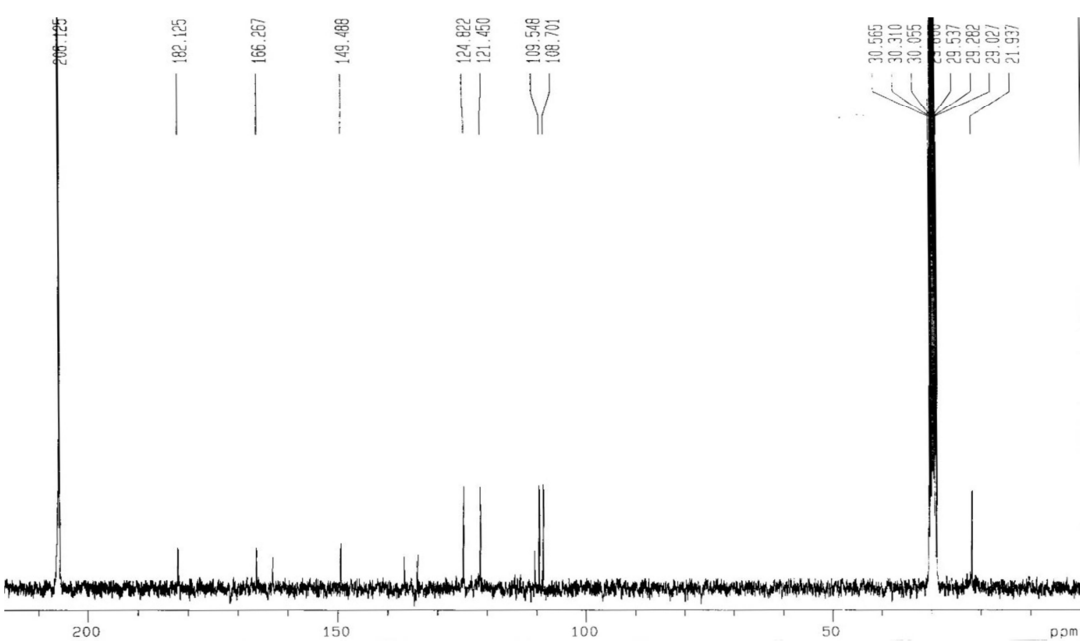


Fig. 9. ^{13}C-NMR spectrum of compound 3 (75 MHz, acetone-d_6)

3.1.4. Structure determination of compound 4

Compound **4** was isolated as yellow powder with the molecular formula $C_{14}H_{12}O_5$. Its IR, UV-Vis spectra, and the presence of one peri-hydroxy group (12.60, 1H, s) indicated a juglone derivative, a napthoquinone.[43] The ^{1}H NMR spectrum of compound **4** showed a series of singlets corresponding to aromatic methyl (2.33, s), acetyl methyl (2.53, s), methoxy (3.97, s), a peri-hydroxy (12.60, s), and an isolated aromatic and a quinonoid proton (Table 2), These data reminisced stypandrone [44], except for the methoxy group. Comparison of its ^{1}H and ^{13}C NMR data with published data[4] give us to conclude that compound **4** is 2-methoxystypandrone, a compound was also previously isolated from the root of this plant [45].

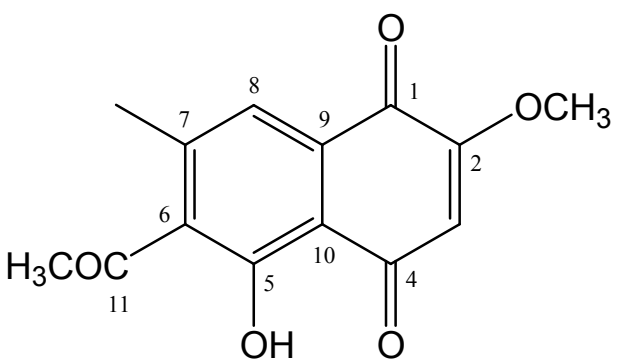


Figure 10: Chemical structure of 2-methoxylstypandrone (4)

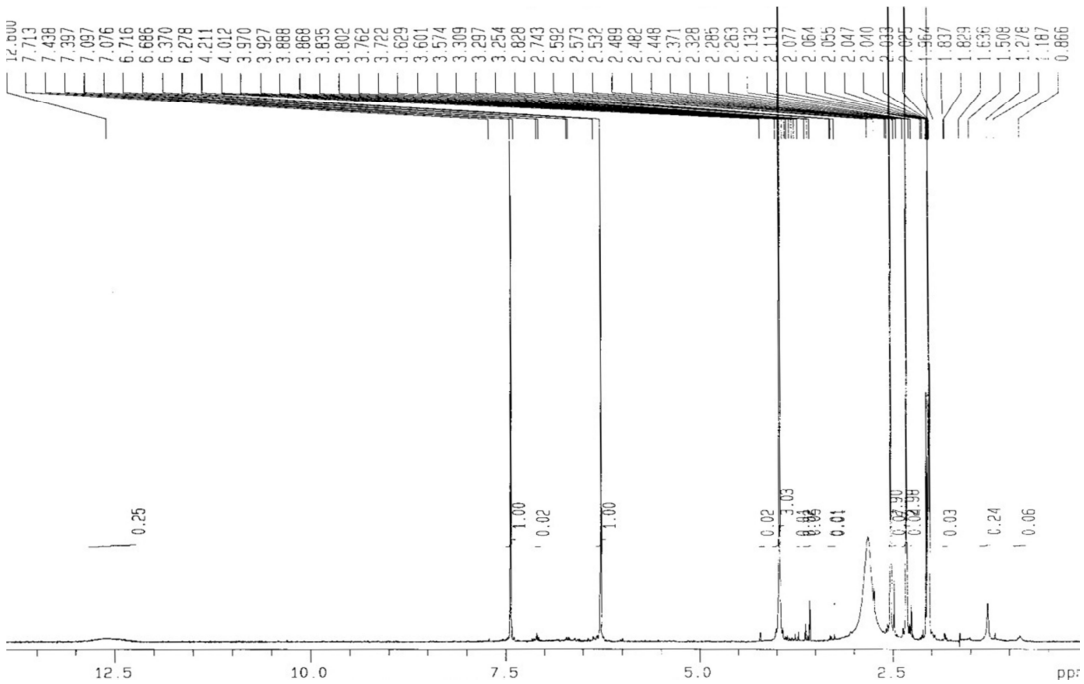


Fig. 11. ^{1}H-NMR spectrum of compound 4 (300 MHz, acetone-d_6)

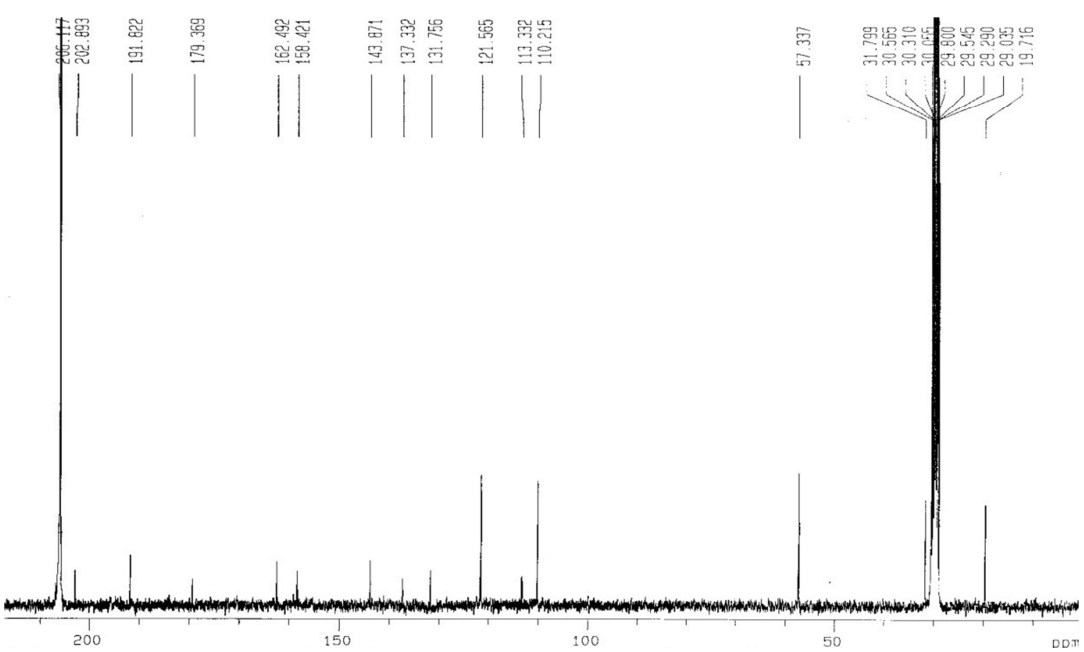


Fig. 12. ^{13}C-NMR spectrum of compound 4 (75 MHz, acetone-d_6)

3.1.5. Structure determination of compound 5

Compound **5** were isolated as white amorphous powders. Comparison of the ^{1}H and ^{13}C NMR spectra of compounds **5** with published data revealed that compounds **5** was resveratrol [46]. This known compound is presented in many plants such as grape. In the previous study, we have also isolated this compound from *Vitis amurensi* [47] and *Vitis vinifera* [46]. Here, we showed the comparison of resveratrol (**5**) by 1D NMR spectroscopic data for confirming the chemical structure of this compound.

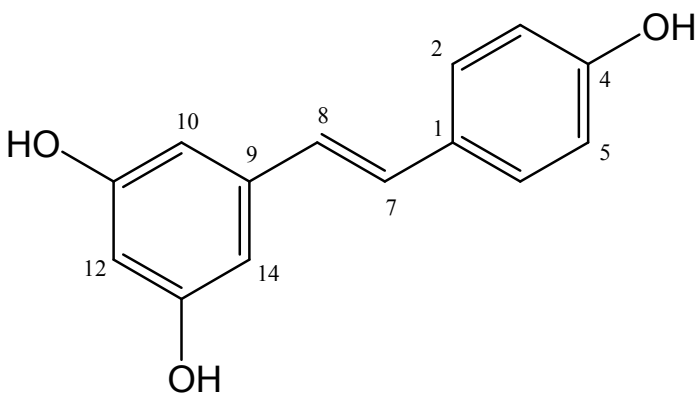


Figure 13: Chemical structure of resveratrol (5)

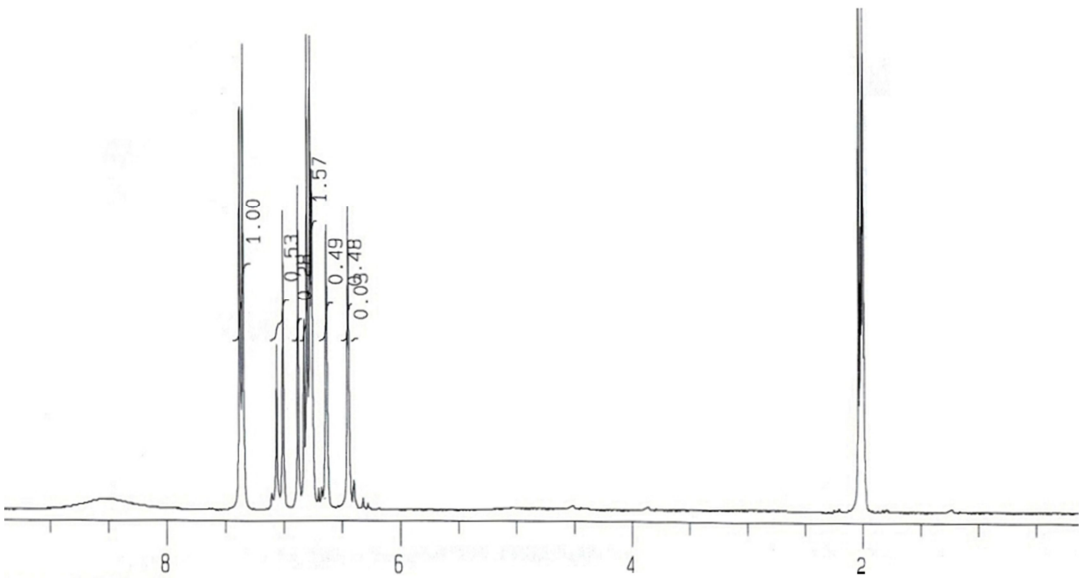


Figure. 14. ^{1}H-NMR spectrum of compound 5 (300 MHz, acetone-d_6)

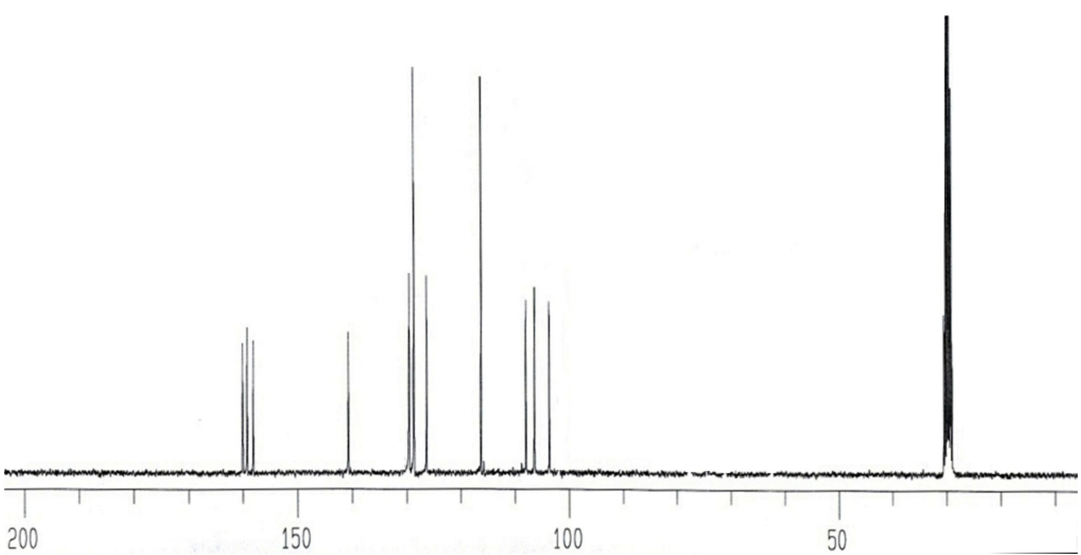


Figure. 15. ^{13}C-NMR spectrum of compound 5 (75 MHz, acetone-d_6)

Table 1. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR data of isolated compounds **1**−**5** from *P. cuspidatum* in acetone-d_6

position	**1**		**2**		**3**		**4**		**5**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	162.5		162.5		162.5		179.4		127.7	
2	124.5	7.05, d, 1.5	121.2	7.26, br, s	124.8	7.08, br, s	162.5		128.7	7.25, br, d, 8.0
3	148.4		152.5		149.5		110.2	6.28, 1H, s	115.3	6.73, br, d, 8.0
4	121.3	7.60, d, 1.5	119.9	7.69, br, s	121.5	7.50, br, s	191.8		157.7	
5	108.2	7.33, d, 2.7	117.7	7.74, dd, 1.5, 8.4	108.2	7.19, d, 2.4	158.4		115.3	6.73, br, d, 8.0
6	166.5		136.9	7.60, br, d, 8.4	166.3		131.7		128.7	7.25, br, d, 8.0
7	106.7	6.65, d, 2.4	124.5	7.22, dd, 1.5, 8.4	106.8	6.60, d, 2.4	143.9		129.5	7.00, d, 16.0
8	165.2		162.0		165.2		121.6	7.44, 1H, s	126.3	6.99, d, 16.0
9	190.8		192.4		191.1		137.3		138.0	
10	182.0		182.0		182.1		113.3		104.8	6.65, br, s
11	110.2		115.7		110.6		202.9		160.1	
12	135.2		133.4		135.3				102.8	6.05, br, s
13	113.7		114.9		113.9				159.3	
14	135.2		133.3		135.5				104.1	6.45, br, s
1-OH		12.29, s		12.12, s		12.29, s				
5-OH								12.60, 1H, s		
9-OH		12.09, s		12.11, s		12.09, s				
2-OMe							57.3	3.97, 3H, s		
7-OMe	56.1	3.91, s								
3-Me	22.2	2.43, s			21.9	2.41, s				
7-Me							19.7	2.33, 3H, s		
3-CH_2OH			63.1	4.65, s						

3.2. Effect on SIRT1 activation of isolated compounds from *P. cuspidatum*

3.2.1. *In vitro* screening of KMP using a luciferase assay

We have used a SIRT1-p53 luciferase assay to investigate nearly one thousand Korean medicinal plants extracts for their activity on SIRT1. After three plant extracts were combined to make a group (TCMU, TCU), these mixture units were screened for extract groups that exhibited potentially stimulation on SIRT1 activity (**Fig.16**). And then, we screened for after divided as single plant extract of candidate mixture groups with the activation effect on SIRT1 (**Fig.17**). From these screening procedures, we can get the candidates with potential activation of SIRT1.

3.2.2. Cell toxicity of isolated compounds

After 293T cells were treated by the tested compounds of 10 μM for 12 hours, cell viability was measured by MTT assay in (**Fig.18**). Compounds **1 - 5** were not toxic to 293T cell compared to non treated cells. The cytotoxicity results are expressed as the mean ± standard deviation Error bar = SD value.

3.2.3. Effects of the compounds on SIRT1 deacetylation activity

Since compounds **1 - 5** exhibited a decrease in p53 transcriptional activity, we further confirmed their direct effects on SIRT1 deacetylation activity by determining the real level of substrate used in the SIRT1 reaction using an NAD/NADH assay. The known SIRT1 activator, resveratrol, was used as a positive control in this assay. As shown in **Fig.22** and **23**, these compounds strongly stimulated SIRT1 deacetylation activity by a dose-dependent manner by comparison with the control.

3.2.4. Effects of the compounds on SIRT1 activity

P53-induced luciferase activity from p53-luc was reduced by SIRT1. To further address the effect of the compounds on SIRT1 activity, 293T cells were transfected with reporter plasmids containing p53-Luc or SIRT1 and pRL-SV plasmid. Cells were then treated with 10 μM compounds and 10 μM Resveratrol. Compound 1-5 strongly decreased p53 transcription activity compared to the control (**Fig 19, 20**).

3.2.5. Compound on the NAD-to-NADH Ratio

We measured the NAD/NADH ratio in 293T cells treated with compound **1 - 5** for 12h. As shown in (**Fig.21)**, compound increased the NAD/NADH ratio. NAD/NADH ratio in 293T cells were increased by compound **1 - 5**, that further support the catalytic activity of SIRT1 in vivo.

3.2.6. Decreased of β–galactosidase activity in HDF cells by the compounds

We measured the β-galactosidase activity in senescent HDF (human diploid fibroblast) cells treated with compounds **1** – **5** at 5 μM concentration. Compound **1** – **5** decreased the β-galactosidase activity as compared to control. Further, compound **4** reversed the senescence after compound **4** (**Fig.24**). Taken together, these data suggest that compounds activate SIRT1 in HDF cells.

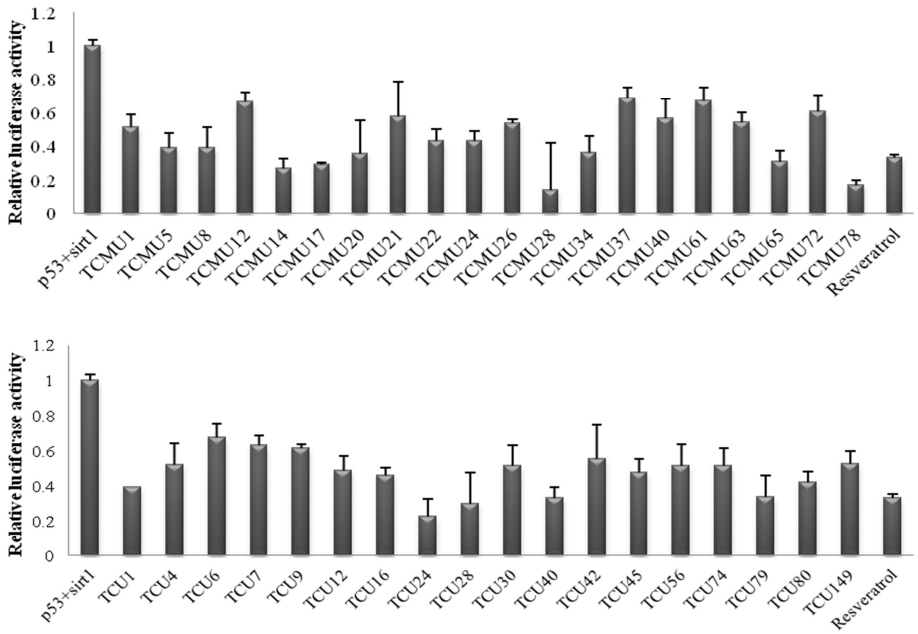


Figure. 16. Screening for mixture extracts (from three plants) with the activation effect on SIRT1

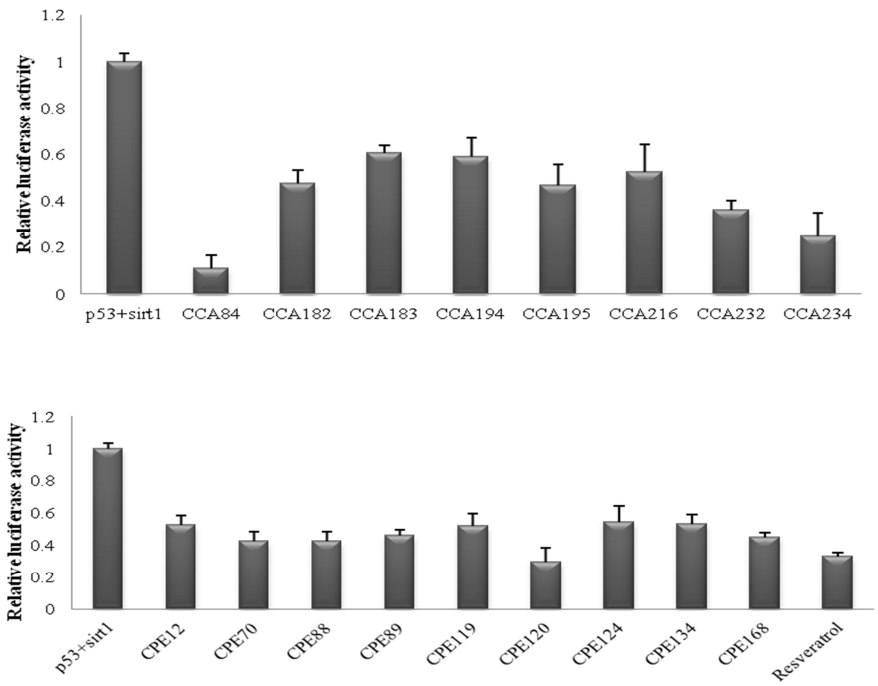


Figure. 17. Screening for single plant's extract with the activation effect on SIRT1

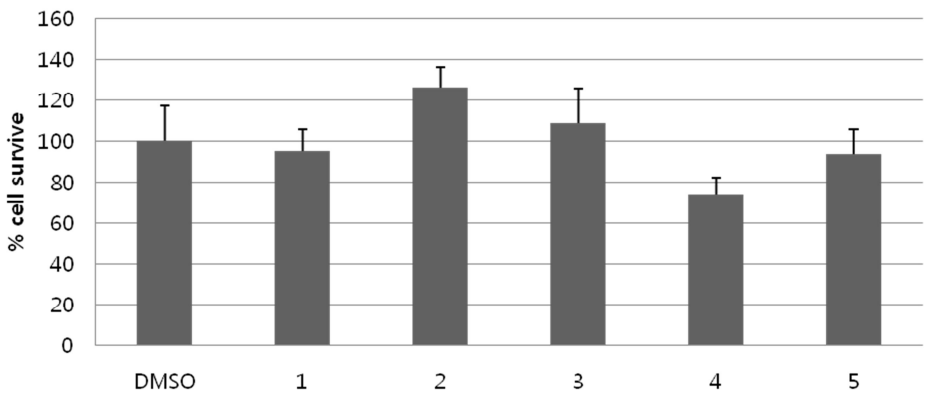


Figure. 18. Compound 1 - 5 on the cell viability in 293T cells. Cell viability was evaluated by MTT assay 12h after compound treatment (10 μM) in 293T cells . Data represent the mean ± SD of three independent experiments

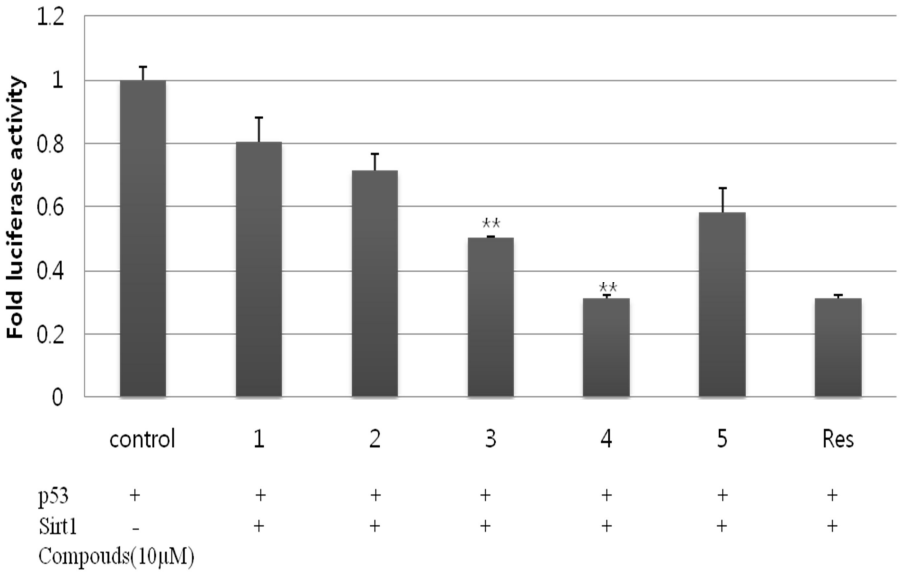


Figure. 19. Effect of compounds 1 – 5 on SIRT1 activation in 293T cells , luciferase activity were exposed to compound (10 μM) for 12h and Resveratrol (10 μM) used as positive control for 12h , DMSO was used as a vehicle (control). Error bar expresses duplicate experiment. ** $P < 0.005$

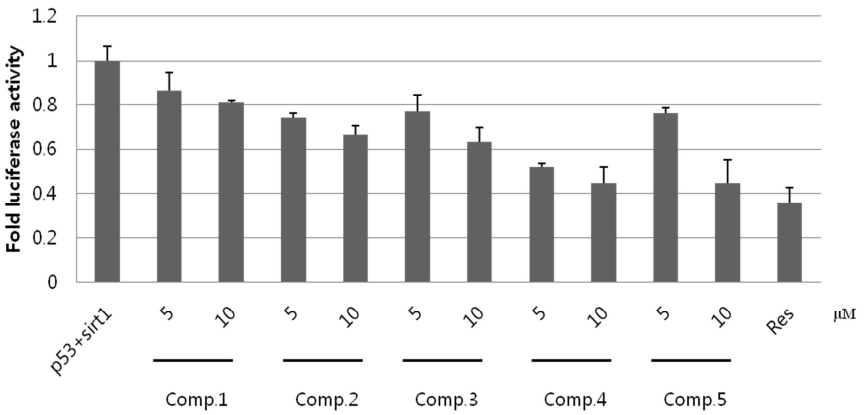


Figure. 20. Effects of compounds 1 - 5 on activity of SIRT1 with different concentration

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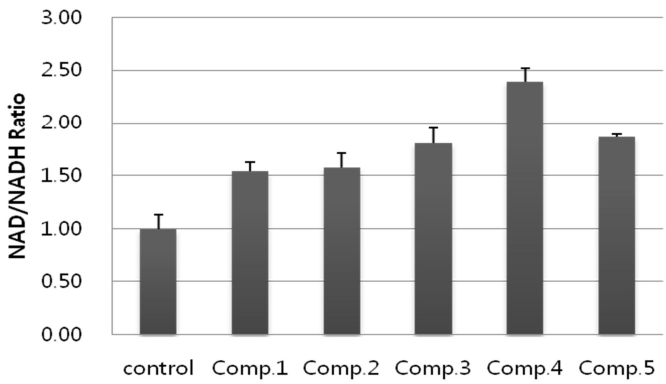

Figure. 21. NAD-to-NADH Ratio in 293T treated with vehicle(-) or compound for 12h at 10 μM

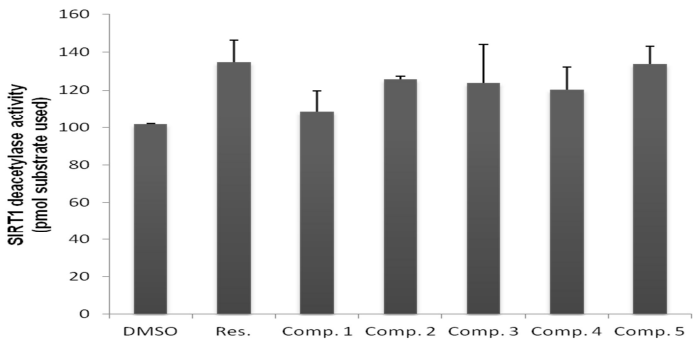

Figure. 22. SIRT1 deacetyl activity with comounds 1 - 5 at 10 μM , positive control used resveratrol

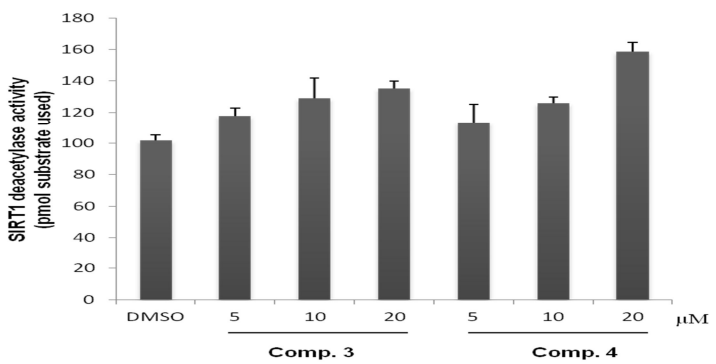

Figure. 23. SIRT1 deacetylase activity of compounds with different concentration

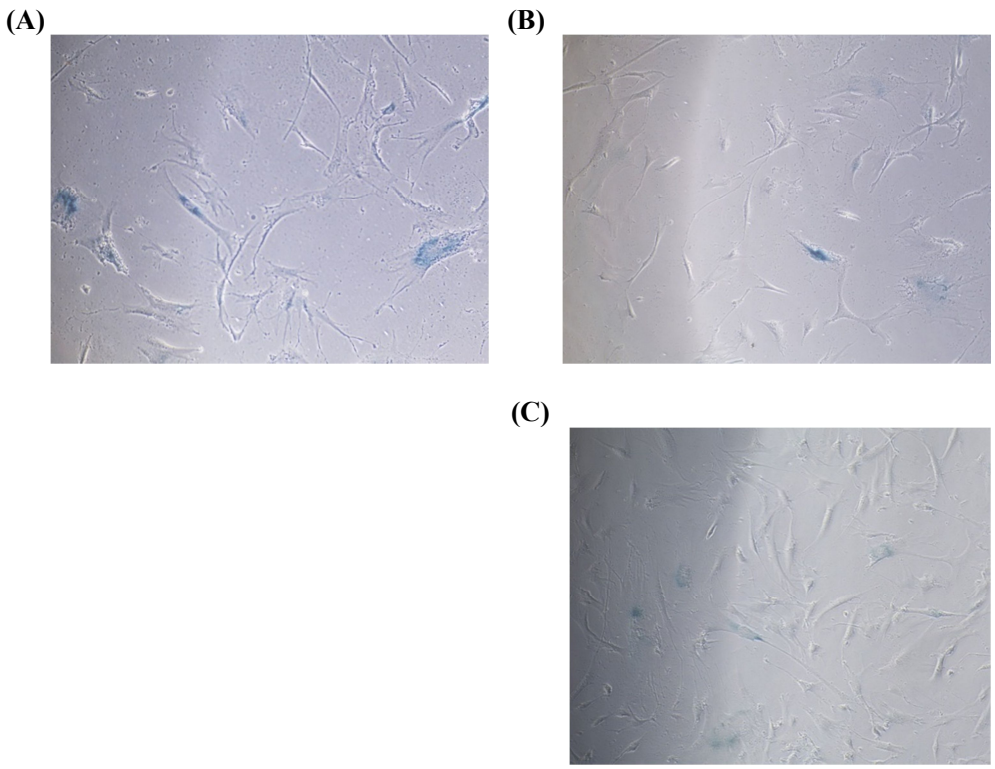

Figure. 24. β–galactosidase activity in the HDF cells, The cells were treated with compounds 4 at 5 µM (B) and 10 µM Resveratrol (C), compared with non treated cells (A).

3.3. Discussions

Previous studies have showed that small compounds isolated from natural products play a key role in anti-aging effect as a Sirt1 activator. These findings may be used in important biomedical therapy for the treatment of age-related disorders. In this research we further found that our compounds repress p53 activity and increase SIRT1 activity, preventing p53 dependent cellular senescence. Based on these findings we can conclude that SIRT1 p53 axis is also involved in determination of cell growth or senescence, in response to various types of stress in mammalian cells [48]. *In-vitro* testing of the isolated compounds **1** – **5** showed that these anthraquinones significantly increase Sirt1 activity. After co-transfection of 293T cells with p53-luc, p53 and Sirt1, p53 transcription activity was strongly decreased by the compounds. Also SIRT1 deacetylation activity was increased in a dose-dependent manner by the compounds in *in-vitro* assay and also increased the NAD-to-NADH ratio. Mainly, compound **4** increased SIRT1 expression and its enzymatic activity and also changed cell's morphology. Further, this compound decreased the β–galactosidase activity in senescent HDF (human diploid fibroblast) cell lines.

Many studies have been reported that Sirt1 activators improve glucose homeostasis and insulin sensitivity in key metabolic tissues like muscle, liver and fat. Sirt1 is also known to play an important role during myocyte differentiation. Thus, our results indicate that Sirt1 activators can be used as a role of useful medication, key target to lifespan extension and treat many other diseases also.

4. Conclusions

It has been shown that SIRT 1 activators might be beneficial in inhibition of aging, diabetes and obesity. Although several SIRT 1 substrates have been characterized, but very few activators have been studied and further exploration is still required. Therefore, it is strongly suggested that if we find the new SIRT1 activators, they can be useful in the treatment of diabetes, obesity and as anti-aging agents. So, more new activators are still needed to improve the understanding of SIRT1 biological functions. Since plants are a promising source for the development of new SIRT1 activators, we have screened one thousand Korean medicinal plant's extracts to find Sirt1 activator.

In the course of *in vitro* SIRT1 activity screening on various extracts from medicinal plants, a methanol-soluble extract of *polygonum cuspidatum* was found to activate SIRT1. Bioassay-guided fractionation of the MeOH extract of the root of *polygonum cuspidatum* has resulted in the isolation of five anthraquinone using chromatography methods (silica gel, C-18, and preparative HPLC). As the active constituents, Physcion, Aloe-emodin, Emodin, 2-methoxystypandrone, Resveratrol were isolated from this extract. Their structures were elucidated on the basic of spectral (including 1D, 2D-NMR, UV, IR, and MS) and physicochemical analyses. The activities of test compounds on SIRT1 expression and its enzymatic activity were also evaluated. The experimental results showed that SIRT1 were activated by compound **1** - **5**, compound **4** showed the strongest effects on SIRT1 activity. This active compound can be used as a potential Sirt 1 enhancing constituent.

5. References

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김 자 연

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본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

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

2012 년 8월 24일

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