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백렴으로부터 항산화 물질의 분리와 자외선이 조사된 사람 섬유아세포의 Matrix Metalloproteinase-1 발현에 미치는 영향

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Antioxidant Constituents from *Melothria heterophylla*; Regulation of Matrix Metalloproteinase-1 Expression in Ultraviolet A-irradiated Human Dermal Fibroblasts

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요 약: 노화에 미치는 자외선의 영향에 대해 많은 연구가 진행되어 왔지만, 천연물에 대한 연구는 별로 알려진 것이 없다. MMPs는 광노화 과정에 매우 중요한 역할을 하는 것으로 알려져 있다. 본 연구에서는 MMP-1의 발현과 활성 및 항산화 효과에 미치는 1,2,4,6-tetra-*O*-galloyl-β-$_D$-glucopyranose와 3,4,5-trihydroxybenzoic acid의 효과를 측정하였다. 이들 화합물은 박과의 백렴으로부터 분리하였으며, 자유 라디칼과 활성산소 소거활성이 매우 높은 것으로 나타났다. 이들 화합물의 DPPH 라디칼과 활성산소를 50% 소거하는 활성(SC_{50})은 각각 3.9 μM, 13.3 μM, 4.3 μM, 4.0 μM로 나타났다. 또한, 이들 화합물은 단백질 수준에서의 MMP-1 발현 및 활성을 처리농도 의존적으로 저해하였지만, 유전자 수준에서는 저해활성이 없는 것으로 나타났다. 따라서, 이들 화합물은 단백질 수준에서의 우수한 MMP-1 발현 저해능과 높은 항산화 활성을 가지는 것을 알 수 있다. 결론적으로 이들 화합물은 새로운 항노화 소재로 적용될 수 있을 것으로 기대된다.

Abstract: Although many studies have been performed to elucidate the molecular consequence of ultraviolet irradiation on an aging, little is known about the effect of natural products. Matrix metalloproteinases (MMPs) are known to play an important role in (a) photoaging. Here we investigated the effect of 1,2,4,6-tetra-*O*-galloyl-β-$_D$-gluco- pyranose (1) and 3,4,5-trihydroxybenzoic acid (2) on the expression of MMP-1 in UVA-irradiated human skin fibroblasts (products), (on the) activity of MMP-1, and (on the) scavenging activities of free radicals. Compounds 1 and 2 were isolated from *Melothria heterophylla* (Cucurbitaceae). These compounds were found to scavenge free radicals and reactive oxygen species (ROS) and were measured to have the SC_{50} values of 3.9 μM and 13.3 μM against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and 4.3 μM and 4.0 μM against superoxide radicals in the xanthine/xanthine oxidase system, respectively. Compounds 1 and 2 showed a dose-dependent inhibitory effect on the expression and activity of MMP-1 in the UVA-irradiated human skin fibroblasts. Therefore, we concluded that compounds 1 and 2 significantly inhibited MMP-1 expression at the protein level. Also, these compounds were determined to have a potent antioxidant activity. From these results, we suggest that these compounds may be used as (a) new anti-aging agents for the photo-damaged skin.

Keywords: *Melothria heterophylla, antioxidants, UVA irradiation, MMP-1, skin aging*

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases that are capable of degrading almost all of the components of the extracellular matrix (ECM) and classified in more than 20 species. MMPs can be divided into four categories based on substrate specificity: collagenases, gelatinases, stromelysins, and membrane-associated MMPs [1]. The expression of MMPs in UV-irradiated fibroblasts is

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known to be initiated by reactive oxygen species (ROS) and by activation of a cell surface growth factor and cytokine receptors. With increasing age, collagen synthesis becomes lower and MMP-1 levels become higher in sun-protected human skin *in vivo*. UV irradiation and ROS induces the synthesis of MMPs in skin fibroblasts *in vitro* and MMP-mediated collagen destruction accounts, in large part, for the connective tissue damage that occurs in photoaging [2].

Melothria heterophylla, the family Cucurbitaceae has been traditionally used for the treatment of the conjunctivitis, an orchitis, skin eczema, and tubercle of a lymphatic gland.

This study deals with the isolation and structure identification of compounds from the roots of *M. heterophylla*, as well as the evaluation of antioxidant effect and inhibitory effects of these compounds on the MMP-1 expression in the UVA-irradiated human dermal fibroblasts.

2. Materials and Methods

2.1. General

M. heterophylla was purchased at a herbal market in Korea. The organic solvents and chemicals were purchased from Sigma-Aldrich Co., Bio-Whittaker, and Gibco BRL (USA) and purified by the appropriated method before use.

2.2. Measurement of Antioxidant Activity

The scavenging effect of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was evaluated according to the method of Hatano *et al.* [3] with minor modification. And the scavenging activity on the ROS from xanthine/xanthine oxidase system was measured by monitoring the reduction of nitroblue tetrazolium (NBT).

2.3. Collagenase (MMP-1) Inhibition Assay

The *in vitro* collagenase inhibition assay was performed using EnzChek Collagenase/Gelatinase kits (Molecular Probes Inc., USA) according to the supplier's instruction. The enzymes were mixed with quenched fluorescent substrates (250 mg/mL) in a final volume of 200 mL of reaction buffer in 96-well microplates. Digested products from DQ collagen substrates have absorption maximum at 495 nm and fluorescence emission maximum at 515 nm in a LS55 fluorescence microplate reader (Perkin Elmer, USA).

2.4. Cell Culture and UVA Irradiation

Human dermal fibroblasts (HDFs) were purchased from Modern Tissue Technology Inc. (Korea) and maintained in DMEM/F-12 (3 : 1) supplemented with 10% FBS and 1% penicillin-streptomycin. HDFs were grown at 37℃ in a CO_2 incubator and irradiated at a distance 15 cm from the UVA source (F15T8.BLB, Sankyo Denki, Japan), filtered for the emission of UVA (320 ~ 400 nm). The dose of UVA radiation was set at 6.3 J/cm^2.

2.5. Evaluation of MMP-1 Synthesis by Enzyme Linked Immunosorbent Assay (ELISA)

After UVA irradiation, HDFs were cultured with serum free medium containing samples for 24 hrs. Then, the supernatants were transferred to a 96-well plate and coating buffer was added with the same volume and incubated for 24 hrs. The expression level of MMP-1 was determined by ELISA method. Finally, cytotoxicity of the supplemented samples was measured by the MTT assay [4].

2.6. Extraction and Isolation

The dried roots (466 g) were refluxed with 70% aqueous ethanol and the solvent was removed by the evaporation. The extract (84.4 g) was suspended in water and the suspension was partitioned with hexane, CH_2Cl_2, EtOAc (2.7 g), and butanol, consecutively. The EtOAc extract was subjected to a Sephadex LH-20 CC (55 × 4.5 cm) with a gradient of MeOH and H_2O (40% ~ 100%) to give 20 fractions (1 ~ 20). Compound **1** (370 mg) was obtained by recrystallyzation in EtOAc from fraction 15. Fraction 12 was further separated by a Sephadex LH-20 CC (35 × 2.3 cm) eluted with Hexane-EtOAc-MeOH (7:3:1 ~ 4:6:4) to yield 9 subfractions (I ~ IX). Compound **2** (105 mg) was obtained from subfraction VIII. Compounds **1** and **2** were identified using ^{13}C-, ^{1}H-NMR spectrum (solvent; Acetone-d_6) and FAB-MS (JMS-700, Jeol, Japan).

2.7. Statistical Analysis

The statistical significance of the results was analyzed by Student's *t*-test for unpaired observations.

Table 1. Antioxidant Effects of Compounds **1** and **2** Isolated from *M. heterophylla*

Compounds	SC_{50}[a] values (mM)	
	DPPH[b]	Superoxide anion[c]
1	3.9	4.3
2	13.3	4
Vitamin C*	60	ND[d]
Vitamin E*	30	ND[d]
EGCG*	6.4	4.9
BHA*	ND[d]	180

[a] Concentration giving a 50% decrease of DPPH and superoxide radicals. The values are the means of triplicate experiments with SD.

[b] 1,1-diphenyl-2-picrylhydrazyl radical

[c] Superoxide anion radicals were produced from xanthine/xanthine oxidase oxidation system.

[d] Not determined

* Used as a positive control

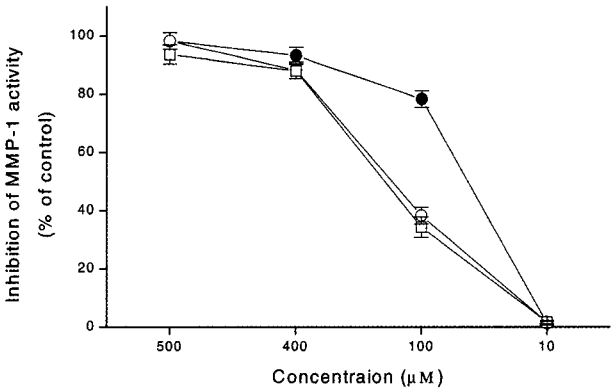


Figure 1. Inhibitory effects of compounds **1** and **2** isolated from *M. heterophylla* on collagenase (MMP-1) activity. Fluorometic assay of the activities of MMP-1 was performed in the presence of increasing concentrations of 1,10-phenanthroline (□), compound **1** (●), and **2** (○). The results were expressed as mean values (± S.D.) of triplicate experiments.

3. Results and Discussion

3.1. Antioxidant Activity

Compounds **1** and **2** exhibited a potent scavenging activity against the DPPH radicals with the SC_{50} values of 3.9 mM and 13.3 mM, respectively. Furthermore, compound **1** appeared to be the most efficient in comparison with the three reference compounds, vitamin C, E, and epigallocatechin-3-gallate (EGCG), which are well known as scavengers of DPPH radicals (Table I). Also, compounds **1** and **2** exhibited a potent scavenging activity against the superoxide radicals in the xanthine/xanthine oxidase system with the SC_{50} values of 4.3 mM and 4.0 mM, respectively, compared with butylated hydroxyanisole (BHA) and EGCG, which are well known as scavengers of superoxide radicals (Table 1).

3.2. Inhibitory Effect of Compounds 1 & 2 on MMP-1 Activity

Hantke *et al.* [5] have reported that flavonoids and vitamins reduced the MMP expression at the protein levels and the MMPs activity in the skin after sun exposure. We first studied the inhibitory effect of these compounds isolated from *M. heterophylla* on *in vitro* collagenase activity. 1,10-phenanthroline, which is well known as a metal chelator and general inhibitor of MMPs, was used as a control to optimize conditions for screening potential gelatinase or collagenase inhibitors. Compounds **1** and **2** were found to have a potent and distinct inhibitory activity against MMP-1 with the IC_{50} values of 60.0 mM, 100.0 mM, respectively (Figure 1). These compounds were inhibited MMP-1 activity in a dose-dependent manner. Especially, compound 1 showed a 2-fold higher inhibitory effect for MMP-1 activity than that of 1,10-phenanthroline.

3.3. Effects of Compounds 1 & 2 on the Expression of MMP-1

The effect of these compounds on the viability of HDFs was investigated for photo-protective activities on the protein levels by the MTT test. These compounds did not show cytotoxicity against HDFs in tested dose compared to control (Figure 2).

We further investigated the inhibitory effects of these compounds on the MMP-1 expression in UVA-irradiated HDFs (6.3 J/cm^2). HDFs were treated with various concentrations of these compounds for 24 hrs and then, the MMP-1 content in the culture medium were determined by ELISA. The treatment of UVA-irradiated HDFs with these compounds significantly suppressed MMP-1 production at the protein levels in a dose dependent manner (Figure 2). Interestingly, the inhibitory effect of compound **1** on MMP-1 production at the protein levels was significantly higher than that of all-*trans* retinoic acid (*t*RA), which is well known

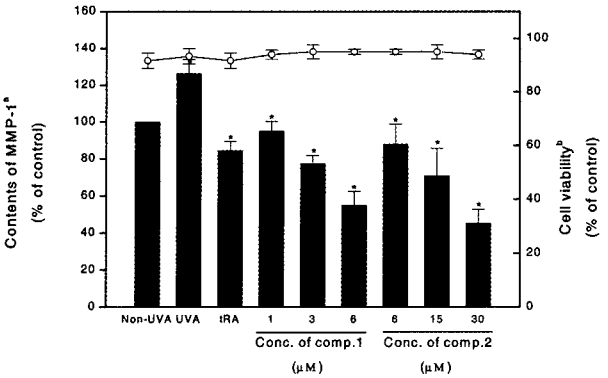
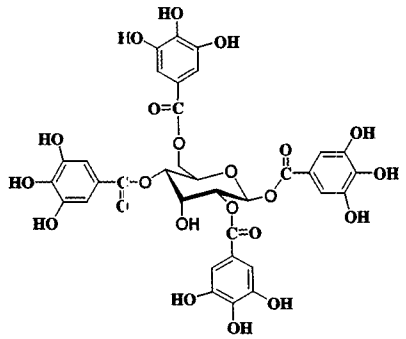


Figure 2. Effects of compounds 1 and 2 isolated from *M. heterophylla*on the production of MMP-1 in UVA-irradiated human dermal fibroblasts. The cells were treated with various concentrations of these compounds for 24 h. [a]MMP-1, matrix metalloproteinase-1 (■). The MMP-1 content in culture media was determined by ELISA as detailed under the Materials and Methods. [b]Cytotoxicity was measured by MTT assay (○). The results were expressed as the average of triplicate experiments with SD. $*p < 0.05$ compared with UVA-treated control cell. UVA dosage was 6.3 J/cm^2 tRA refers to 4.0 mM of all-*trans* retinoic acid.

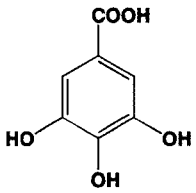
as an inhibitor of UVA-induced MMPs. Fisher *et al.* [6] have reported that *t*RA, which trans-represses transcription factor (AP)-1, substantially reduced AP-1 and MMPs induction in human skin.

3.4. Identification of Compounds 1 & 2 Isolated from *M. heterophylla*

Activity-guided column chromatographies of an EtOAc soluble fraction from the extracts of *M. heterophylla* led to the isolation of two compounds. Compound 1 was formed as an amorphous pale brown powder. Its molecular formula was determined to be $C_{34}H_{28}O_{22}$ (*m/z* 789 [M$^+$+1]) by FAB-MS. Its structure was identified as 1,2,4,6-tetra-*O*-galloyl-b-D-glucopyranose(Figure 3) by a comparison with the ^{1}H-, ^{13}C-NMR and MS data reported in the literature[7,8]. Compound 2 was obtained as white crystals. Its molecular formula was determined to be $C_7H_6O_5$ (*m/z* 171 [M$^+$+1]) by FAB-MS. It was identified as a 3,4,5-trihydroxybenzoic acid(Figure 4) on the basis of chemical and physicochemical evidences, and compared its spectral data previously reported in the literature [9].



Compound 1



Compound 2

Figure 3. Chemical structures of compounds 1 and 2 isolated from *M. heterophylla*.

4. Conclusion

Two compounds isolated from the roots of *M. heterophylla* exhibited significant DPPH and superoxide anion radicals scavenging activities. Also, these compounds significantly reduced the activity and expression of MMP-1 at the protein levels in a dose dependent manner. Therefore, we suggest that these compounds may be potential candidates to protect skin aging from UV stimulation.

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References

1. E. J. Bernhard, S. B. Gruber, and R. J. Muschel, Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells, *Proc. Natl. Acad. Sci. USA*, **91**, 4293 (1994).

2. J. Varani, R. L. Warner, M. Gharaee-Kermani, S. H. Phan, S. W. Kang, J. H. Chung, Z. Q. Wang, S. C. Datta, G. J. Fisher, and J. Voorhees, Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin, *J. Invest. Dermatol.*, **114**, 480 (2000).
3. T. Hatano, H. Kagawa, T. Yasuhara, and T. Okuda, Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects, *Chem. Pharm. Bull.*, **36**, 2090 (1998).
4. T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods.*, **65**, 55 (1983).
5. B. Hantke, C. Lahmann, K. Venzke, T. Fischer, A. Kocourek, L. J. Windsor, J. Bergemann, F. Stab, and H. Tschesche, Influence of flavonoids and vitamins on the MMP- and TIMP-expression of human dermal fibroblasts after UVA irradiation, *Photochem. Photobiol. Sci.*, **1**, 826 (2002).
6. G. J. Fisher, S. C. Datta, H. S. Talwar, Z. Q. Wang, J. Varani, S. Kang, and J. J. Voorhees, Molecular basis of sun-induced premature skin ageing and retinoid antagonism, *Nature*, **379**, 335 (1996).
7. B. S. Min, H. K. Lee, S. M. Lee, Y. H. Kim, K. H. Bae, T. Otake, N. Nakamura, and M. Hattori, Anti-human immunodeficiency virus-type 1 activity of constituents from *Juglans mandshurica*, *Arch. Pharm. Res.*, **25**, 441 (2002).
8. I. M. Chang, *Treatise on Asian Herbal Medicines*, 4534, Natural Products Research Institute of Seoul National University, Seoul (2003).
9. I. R. Lee and M. Y. Yang, Phenolic compounds from *Duchesnea chrysantha* and their cytotoxic activities in human cancer cell, *Arch. Pharm. Res.*, **17**, 476 (1994).