The effect of plant extracts on the activity and the expression of MMPs (matrix metalloprotease) induced by UVA

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Keywords : MMP, UVA, Plant extract, ELISA, Zymography, *Tussilago farfara*, *Dicentra spectabilis*

Summary

UV irradiation on a skin brings about the qualitative and quantitative alterations of the extracellular matrix. Repeated-UV irradiation suppressed the synthesis of collagen and activated the expression of the matrix metalloprotease (MMP).

In this paper, on the purpose of development of novel anti-aging agents from natural sources, effects of several natural products on *in vitro* MMP-1 activity and UVA induced MMP-1 synthesis in human dermal fibroblast (HDF) culture were studied. We measured MMP-1 activities by fluorescence assay using gelatin as substrates. As a result, the extract of *Dicentra spectabilis*, and flower buds of *Tussilago farfara* showed strong inhibitory effect. Among them, the extract of flower buds of *Tussilago farfara* and *Dicentra spectabilis* inhibited MMP-1 activity by 92% and 87% at 0.05% (w/v). And UVA induced MMP-1 expression were analyzed by enzyme-linked immunosorbent assay (ELISA) and gelatin-based zymography in HDF culture. The extract of flower buds of *Tussilago farfara* and *Dicentra spectabilis* suppressed the UVA induced expression of MMP-1 by similar level of Vitamin C 200μM at 0.1% (w/v).

These results suggest that the extract of *Dicentra spectabilis*, and flower buds of

Tussilago farfara effectively prevent skin from the UV-induced photoaging. So the extracts are thought to have potential as effective raw materials for anti-aging cosmetics.

Introduction

Besides of many beneficial effects including vitamin D metabolism, ultraviolet (UV) radiation also gives notorious effects.[1] The long term UVA irradation leads to photoaging in facial skin.[2] UV irradiation on a skin brings about the qualitative and quantitative alterations of the extracellular matrix, including substantial breakage, disorganization, and shortening collagen fibrils in skin connective tissue. [2,3] It is well known that a decrease in collagen is shown with photoaging of human skin.[4,5,6,7]

The matrix metalloprotrease (MMP) family is considered to be primarily responsible for matrix degradation.[24] MMPs are responsible for the degradation of several components (collagen fibers, glycosaminoglycans, fibronectin) of the skin extracellular matrix. This degradation is thought to lead to the formation of fine lines and wrinkles and ultimately skin sagging, hallmarks of skin photoaging. [9,10]

Kligman et al. reported that a loss of collagen may arise however, from an acceleration of enzymatic degradation due to MMPs release from UV-induced infiltrating cells, in which case the rate of collagen degradation exceeds the rate of biosynthesis.[8]

There are also natural inhibitor proteins of MMPs, designated TIMP-1 and TIMP-2.[14,15,16,17] Although, TIMPs have been extensively studied to treat various diseases induced by MMPs, their effect has a limitation so far, probably because of a high molecular weight nature.[14,18,19,20] It is reasonable, therefore, to expect that low molecular-weight ingredients that inhibit the activity and suppress the expression of MMPs are more effective.[14]

In this paper, for an attempt to develop materials protecting UV-induced skin damage, 20 plant extracts were evaluated for their inhibitory activities on MMPs.

From the results of these screening procedures, we suggest that the extract of *Dicentra spectabilis*, and flower buds of *Tussilago farfara*, which can suppress the expression of MMPs and inhibit the activities of MMPs, can protect the skin from photoaging.

MATERIALS AND METHODS

Reagents

Tussilago farfara Linne and *Dicentra spectabilis* were obtained from Odae mountain in Korea. Enz collagenase/gelatinase kit for the Collagenase/gelatinase inhibition assays was purchased from molecular probes Co, anti-mouse IgG for the secondary antibody and Sephadex LH-20 were obtained from Sigma chemical Co. Anti-MMP-1 antibody and anti-mouse IgG conjugated with alkaline phosphatase were obtained from Cal- biochem.

Plant extract preparation

An amount of powdered dry plants were soaked in ten times (w/w) of ethyl alcohol for 72hr at room temperature. And the same amounts of the powdered dry plants were dispersed in 10 times (w/w) of distilled water and extracted for 5hr at 50℃ for the water extracts. The ethanol extracts were filtered and concentrated at 40℃ in vacuum evaporator. And, the water extracts were filtered and lyophilized.

MMP-1 (collagenase) activity assays

We measured *in vitro* MMP-1 activities by fluorescence assay using fluoresein-conjugated gelatin as substrates and collagenase purified from *Clostridium histolyticum* is provided with the assay kit to serve as a control enzyme. 140㎕ of diluted sample with buffer and 40㎕ of Clostridium collagenase dilution were transferred into 96-well plates. And, 20㎕ of The fluorescein conjugated gelatin was added into each well. Then, the samples were incubated at room temperature, protected from light, for an appropriate time.

The fluorescence intensity of digested products from substrates was measured at absorption maxima 495㎚, emission maxima 515㎚ in a fluorescence microplate reader (Perkin Elmer, USA). For all the MMPs tested the activities under these conditions were linear for at least 15min. For each time point, correct for background fluorescence by subtracting the values derived from the no-enzyme control.

Culture of Human dermal fibroblast

HDFs were maintained in Dulbecco's Modified Eagle's Media (DMEM) with 10% FBS and kept in a humidified 5% CO_2 atmosphere at 37℃. HDFs from passage 6 to 10 were used in the experiments.

Cell treatment and UVA irradiation

HDFs (1.5×10^5/well) were seeded into 35ø plates and cultured overnight. Prior to UV

irradiation, the cells were washed twice with phosphate buffered saline (PBS). The cells were irradiated from a distance of 15 cm by a UV source (UVA simulator, Jhonsam, KOREA) emitting wavelengths in the range of 340 - 450 nm. UVA irradiation doses were 5J/㎠ and the radiation intensity was measured using UV radiometer (EKO, JAPAN). The culture media, DMEM without serum, containing the plant extracts was immediately added to the cells.

Zymography

Zymography in SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) containing 0.15% gelatin was performed according to the method of Demeule et al.[27] Samples were mixed with SDS sample buffer in the absence of reducing agent, incubated at 37℃ for 24hr, and electrophoresed on 10% polyacrylamide gels at 4℃. After electrophoresis, the gels were washed 5 times with 50mM Tris-HCl, pH 8.0 containing 2.5% Triton X-100 for 30min to remove SDS and incubated for 12h at 37℃ in 50mM Tris-HCl, pH 7.6, 20mM NaCl, 5mM $CaCl_2$, and then stained with 0.1% Coomassie Brilliant Blue R250.

Determination of MMP-1 by ELISA

The expression of MMP-1 and MMP-2 was assayed by enzyme-linked immunosorbent assay (ELISA). HDFs were seeded into 96-well plates and cultured overnight. The culture media were replaced with DMEM containing sample. After 24 hours' incubation, the supernatants were transferred into a 96 well plate and the coating buffer (Na_2CO_3 1.59%, $NaHCO_3$ 2.93%, NaN_3 0.20%, $MgCl_2$ 1.02%, pH 9.6) was added 1:1 (v/v) and incubated for 12 hours. The supernatants were removed and the coated well was washed with PBS-T for 3 times and followed by blocking with 5% BSA in PBS-T for 1 hour at 37℃. After washing 3 times with PBS containg 0.05% Tween 20 (PBST), 100㎕ of diluted primary antibody were added into each well and incubated for 90 min at 37℃. Washing the wells with PBST 3 times, 100㎕ of diluted secondary Ab, anti-mouse IgG conjugated with alkaline phosphatase in PBST was added and incubated for 2hr at 37℃. After 3 times washing with PBST, 200㎕ of p-nitrophenyl phosphate (pNPP) 1mg/ml in 10% diethanolamine buffer, pH 9.8, containing 0.5mM $MgCl_2$ /ml was added. The optical density was measured at 405nm after 30 min.

RT-PCR

RT-PCR was performed using a OneStep RT-PCR Kit(QIAGEN CO.). cDNAs were amplified with Taq polymerase using specific primers for MMP-1 and β-actin. The primer sequences are given below.

MMP1 (sense) 5'-AAAGGGAATAAGTACTGGGC-3'

MMP1 (antisense) 5'-AATTCCAGGAAAGTCATGTG-3'

β-actin (sense) 5'-CGAGCTGCCTGACGGCCAGG-3'

β-actin (antisense) 5'-ATTTGCGGTGGACGATGGAG-3'

Polymerase chain reaction was consisted of cycles of denaturation(94℃, 30sec) ramped annealing(50℃, 1min) and extension(72℃, 1min). Thirty cycles were used for MMP-1 and β-actin. Amplified products were electrophoretically separated in agarose gel containing ethidium bromide and finally analyzed for molecular size.

RESULTS AND DISCUSSION

Screening of plant extracts by the evaluation of inhibitory activities on MMP-1

20 plant extracts were prepared and evaluated for their inhibitory activities on MMP-1. Among them, ethanol extracts of *Dicentra spectabilis, Tussilago farfara L. Ulmus davidiana* showed strong inhibitory effect on MMP-1 above 90% at 0.05%(w/v). In the case of water extracts, *Ampelopsis japonica*, *Dicentra spectabilis* , *Tussilago farfara L.* showed strong inhibitory effect above 90% at 0.05%(w/v). From these results, the extract of *Dicentra spectabilis*, and flower buds of *Tussilago farfara* were selected for further study.

Table 1. The results of screening procedure on the activity of MMP

Plant extracts*	MMP-1 inhibition activity(%)	
	Ethanol extract	Water extract
Alpinia oxyphylla	46	23
Ampelopsis japonica	72	97
Artemisia princeps	30	62
Cassia obustifolia	2	25
Dicentra spectabilis	96	91
Equisetum arvense	0	12
Eriobotrya japonica	0	48
Forsythia suspensa	32	38
Gardenia jasminoides	0	49
Lonicera japonica	8	47
Lycium chinensis	17	34
Oenothera odorata	45	15
Paeonia lactiflora	74	87
Paeonia suffruticosa	54	89
Prunella vulgaris	0	48
Prunus tomentosa	10	11
Saururus chinensis Baill	3	28
Taraxacum mongolicum	0	49
Tussilago farfara L.	94	92
Ulmus davidiana	92	81

* : All plant extracts were dissolved in 5% ethanol at a concentration of 0.05%

Effect of plant extracts on the activity of MMP-1

In vitro MMP-1 activities were measured by fluorescence assay. We found that the green tea extract, known as a general inhibitor of metalloproteinase, showed the inhibition of MMP-1 activity by 94% at 0.02% (w/v). Also, the ethanol extract of *Dicentra spectabilis*, and flower buds of *Tussilago farfara* showed strong inhibitory effect on MMP-1 by 92% and 87% at 0.02% (w/v) as similar level of the green tea extract. But, in the case of the water extract of *Dicentra spectabilis* and flower buds of *Tussilago farfara,* strong inhibitory effect on MMP-1 were not shown at concentration of 0.02% (w/v).

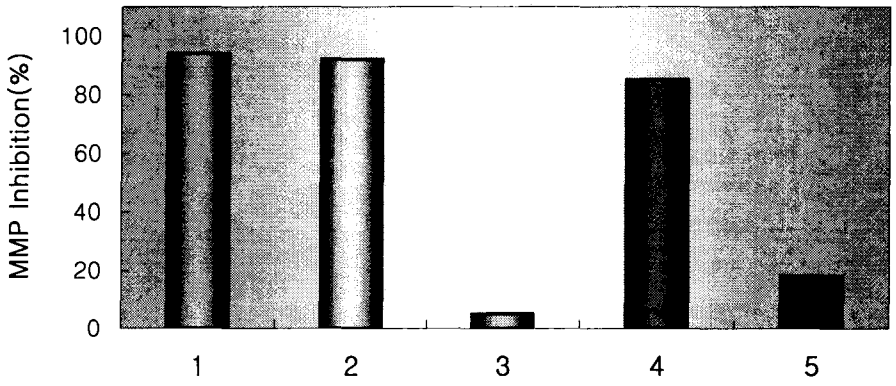

Fig 2. Effects of several plant extracts on the activity of MMP-1

1 *Green tea extract*, 2. *Tussilago farfara* ethanol extract, 3. *Tussilago farfara* H_2O extract, 4. *Dicentra spectabilis* ethanol extract, 5. *Dicentra spectabilis* H_2O extract, Samples were dissolved in 2% ethanol, 0.02%.

Effect of plant extracts on the production of MMP-1 and MMP-2

To quantify protein respectively for MMP-1 and 2 in the culture medium of HDFs and their gelatinase activity were performed by gelatin-substrate zymography. The supernatants of UVA irradiated HDFs showed MMP-1 and MMP-2 activity in SDS-PAGE (lane 1, 7). To determine whether the plant extracts could modulate the production of MMP-1 on UVA irradiated HDFs, each plant extracts was applied for 24hr after UVA irradiation to the cells. In the presence of ethanol extract of flower buds of *Tussilago farfara* and ethanol extract of *Dicentra spectabilis* showed significantly decreased MMP-1 and MMP-2 activity at 0.1% (w/v) (Lane 3, 6) in comparison to UVA irradiated control.

Fig 2. The effects of several plant extracts on the production of MMP-1 and MMP-2 induced by UV irradiation.

1. UVA 5J treated (control), 2. Vitamine C. 3. *Tussilago farfara* ethanol extracts 0.1% 4.*Tussilago farfara* H_2O extract 0.1%, 5. *Dicentra spectabilis* H_2O extract 0.1%, 6. *Dicentra spectabilis* EtOH extract 0.1%, 7. UV 5J treated (control)

Effect of plant extracts on the expression of MMP-1

To evaluate effects of plant extracts on the MMP production from UVA irradiated HDFs, Enzyme-linked immunosorbent assay (ELISA) were used. To determine whether the plant extracts could modulate the production of MMP-1 on UVA irradiated HDFs, each plant extracts was applied for 24hr after UVA irradiation to the cells. In the presence of ethanol extract of flower buds of *Tussilago farfara* and ethanol extract of *Dicentra spectabilis,* the UVA induced expression of MMP-1 was suppressed about 92% and 80% at a concentration of 0.1% (w/v).

But, the water extracts of these 2 plants showed relatively weak effects on the UVA induced expression of MMP-1 at 0.1% (w/v).

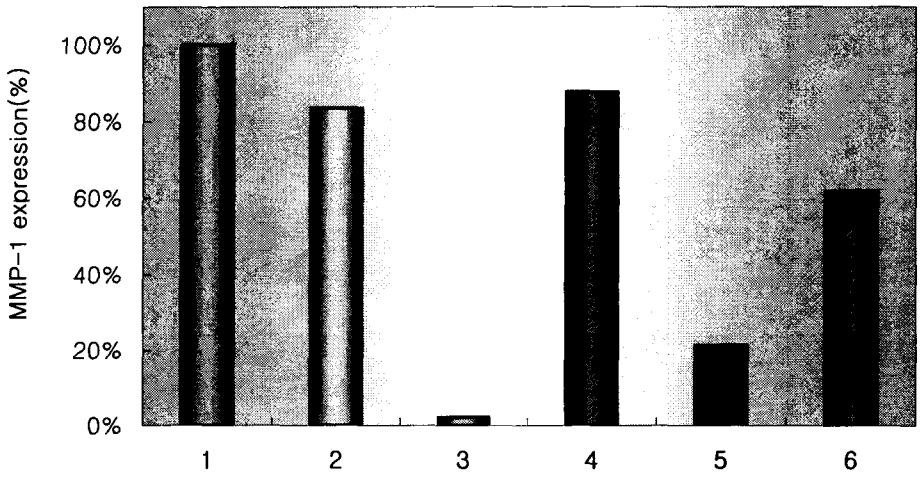

Fig 4. The effects of several plant extracts on the expression of MMP-1 evaluated by ELISA method.

1.UV 5J treated(control), 2.Vitamine C 200μM, 3.*Tussilago farfara* ethaol extract 0.1%, 4.*Tussilago farfara* ethanol H_2O extract 0.1%, 5.*Dicentra spectabilis* ethanol extract 0.1%, 6. *Dicentra spectabilis* H_2O extract 0.1%

Effect of plant extracts on the mRNA expression of MMP induced by UV

The mRNA expression of MMP-1 induced by UVA was analyzed by RT-PCR (Fig-5). These results revealed increased expression of MMP-1 by UVA irradiation. In contrast, mRNA levels of HDFs that treated by the extracts of *Tussilago farfara* showed decreased expression of MMP-1. But, the treatment of the extracts of *Dicentra spectabilis* brought no differences for the mRNA expression induced by UVA irradiation.

These results suggest that the extract of *Tussilago farfara* has inhibitory effects on the UVA induced mRNA expression of MMP-1.

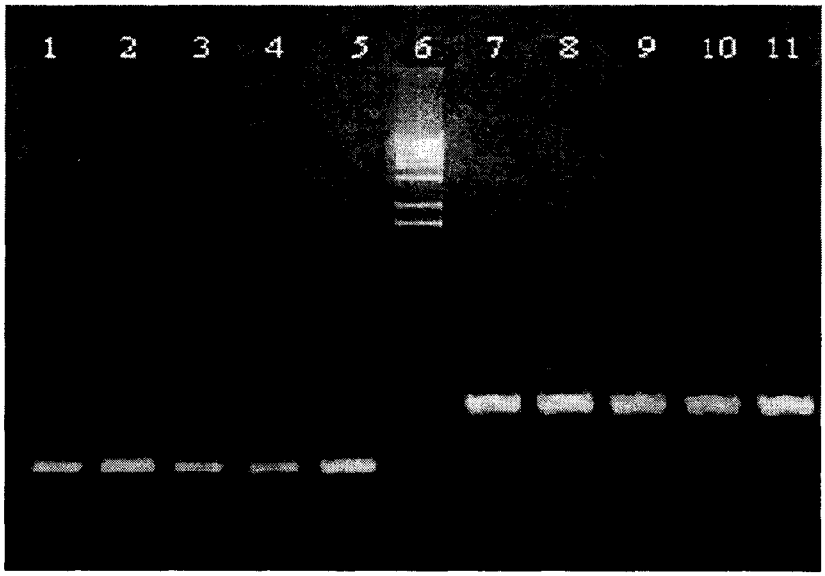

Fig 5. The effect of plant extracts on the mRNA expression of MMP –1 induced by UV irradiation. Lane 1. control(non-UVA irradiated), lane 2. UVA(UVA irradiated), lane lane 3. L-ascobic acid(200uM), lane 4.*Tussilago farfara* ethanol extract, lane 5. Dicentra spectabilis ethanol extract, lane 6. 1kb ladder marker, lane 7~11. β-actin

CONCLUSIONS

On the purpose of development of novel anti-aging agents from natural sources, 20 plant extracts were evaluated for their inhibitory activities on MMPs. From the results of these screening procedures, the extract of *Dicentra spectabilis*, and flower buds of *Tussilago farfara* werer selected for futher study. And, the effects of these natural products on *in vitro* MMP-1

activity and UVA induced MMP-1 synthesis in human dermal fibroblast(HDF) culture were studied.

And, MMP-1 inhibition activity assay by fluorescence method revealed the extract of *Dicentra spectabilis*, and flower buds of *Tussilago farfara* had strong inhibitory effect by 92% and 87% at a concentration of 0.02%(w/v).

And the result of the gelatin-based zymography in HDF culture showed that in the presence of the ethanol extract of flower buds of *Tussilago farfara* and ethanol extract of *Dicentra spectabilis* showed significantly decreased MMP-1 and MMP-2 activity in comparison to UVA irradiated control. Enzyme-linked immunosorbent assay (ELISA) were used to evaluate the MMP production from UVA irradiated HDFs. In the presence of ethanol extract of flower buds of *Tussilago farfara* and *Dicentra spectabilis,* the UVA induced expression of MMP-1 was suppressed about 92% and 80% at a concentration of 0.1% (w/v). The mRNA expression of MMP-1 induced by UVA was analyzed by RT-PCR. mRNA levels of HDFs that treated by the extracts of *Tussilago farfara* showed decreased expression of MMP-1.

These results suggest that the extract of *Dicentra spectabilis*, and flower buds of *Tussilago farfara* have the strong inhibitory activity on MMP-1 activity, and suppressive effect of the UVA induced MMP-1 synthesis.

From these results, the extract of *Dicentra spectabilis*, and flower buds of *Tussilago farfara* are thought to have potential as effective raw materials for anti-photoaging cosmetics.

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