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A Thesis for the Degree of Master of Science

Safflower seed oil and its active compound acacetin protect against UVB-induced skin photoaging in vitro

잇꽃씨유와 그 활성성분인 아카세틴의 자외선에 유도되는 피부세포의 광노화 보호 효능

February 2015

By

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Abstract

Safflower seed oil (*Charthamus tinctorius L.*, SSO) contains a significantly high level of unsaturated fatty acids including oleic and linoleic acids. It has been reported that unsaturated fatty acids repair barrier of skin and nourishes dry skin and hair. There are numerous anti-aging ingredients used to decrease the appearance of wrinkles. However, there is no therapeutic agent that is effective for not only preventing skin wrinkle formation, but also skin hydration. Therefore, it is important to develop a new cosmetic ingredient which is effective for wrinkle prevention as well as skin hydration. For these purposes, I investigated the effect of the SSO and its active compound acacetin on UVB-induced skin photoaging in HaCaT cells and human dermal fibroblasts (HDF). The results show that SSO and acacetin inhibited UVB-induced matrix metalloproteinase (MMP)-1, which plays important role in collagen degradation and wrinkle formation, at both protein and mRNA level in

HaCaT cells and HDF. Furthermore, acacetin suppressed UVB-induced phosphorylation of c-Jun and $p90^{RSK}$ in a dose dependent manner. Taken together, these results show that SSO prevents UVB-induced MMP-1 expression that lead to skin photoaging, and especially its active compound acacetin reduced UVB-induced MMP-1 expression via attenuating phosphorylation of c-Jun and $p90^{RSK}$ signal transductions. Therefore, I suggests that SSO and acacetin have a potential therapeutic anti-wrinkle agent for skin health.

Keywords: Safflower seed oil; Acacetin; UVB; Skin wrinkle; Anti-aging; MMP-1; HaCaT; Human dermal fibroblasts

Student ID: 2013-22559

Contents

I. Introduction

Skin is an outermost organ, one of the principle functions of which is the physical and chemical protection of the body [1]. Skin directly contacts with damaging environmental factors such as sunlight, air pollution, and extreme temperatures, which cause aging of skin [2, 3]. Among the environmental factors, ultraviolet radiation from sun light is one of the most critical factors which leads to accelerated and aggravated skin aging [4]. After irradiation of ultraviolet, skin features deep wrinkles, dry, decreased procollagen production and degradation of collagen, and accelerated decline in the function of skin [5, 6].

Solar ultraviolet (UV) is divided into three subgroups including UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm) [7]. While UVA and UVB reach the surface of earth, UVC

is effectively filtered out by ozone layer [8]. UV energy that reaches the surface of earth is accounted for 90-99% by UVA and contributed the other 1-10% by UVB [9]. UVB contains more energy than UVA, and it is responsible for numerous biological effects on human skin such as sunburn, sun tanning, immune-suppression, and photo carcinogenesis [10]. The use of skin care products supplemented with several effective agents working thorough different pathways in conjunction with the use of anti-wrinkle ingredients may be an effective approach for reducing UVB mediated photoaging [11, 12].

Skin wrinkles are the most prominent characteristic of aging. When collagenase increase and collagen is degraded, skin dermis is collapsed and wrinkles appear. Matrix metalloproteinases (MMPs) are collagenase that are responsible for the degradation or synthesis inhibition of collagenous extracellular matrix (ECM) in connective tissues [13, 14]. Collagen is the major structural component of the

ECM of dermal connective tissue, and its concentration decreases by photoaging [15]. MMP-1 (collagenase), MMP-2 (gelatinase), and MMP-9 (gelatinase) are the principal neutral proteinases capable of degrading native fibrillar collagens in the human skin [3, 16, 17]. Among those MMPs, MMP-1 preferentially degrades fibrillar collagens. Repeated exposure of UVB increases MMP-1 expression in dermis and epidermis tissue [18]. Furthermore, increased MMP-1 expression consequently forms skin wrinkle, therefore inhibition of MMP-1 expression plays a crucial role for preserve collagen in skin. Therefore, MMP-1 is used for major marker of UVB-induced skin photoaging [16, 19].

Recently, many natural compounds have gained attention as anti-aging agents for cosmetics. Retinoic acid, derivative of vitamin A, shows strong protective effects against UV-induced skin wrinkle in human and is used for treatment of skin wrinkles [20, 21]. However,

retinoic acid is easily degraded by light and has phototoxic potential where topical retinoic acid therapy can induce inflammation like retinoid dermatitis [22, 23]. These adverse effects restrict the usage of retinoic acid as a cosmetic ingredient, which requires development of alternative ingredients of anti-aging.

Safflower (*Carthamus tinctorius L.*) is widely distributed in eastern and western Asia, and it have been grown for a long time for oil production and coloring purposes. The flower of safflower is used in folk medicine as an analgesic, antithrombotic and antihypertensive crude drug. Additionally, safflower seed oil (SSO) is rich in unsaturated fatty acids such as linoleic or oleic acid [24]. It has long been used for industrial purposes as supplies oil, meal, birdseed, and cosmetic base ingredients, notably for preparing varnish [25-27]. Recent studies revealed that high-linoleic SSO have been shown to improve lipid metabolism and reduce blood cholesterol level [28, 29],

and to protect against bone fracture and loss [30, 31]. Acacetin (5,7-dihydroxy-4′-methoxyflavone) is a flavonoids isolated from safflower seeds, plants and leaves [32]. Acacetin is shown to exert anti-peroxidative, anti-inflammatory, anti-plasmodial and anti-proliferative activities [33, 34]. Although a broad range of biological and pharmacological activities of SSO and its active phenolic compound acacetin have been reported, anti-wrinkle and photoaging effects have not been investigated [35, 36].

In this study, I reported that safflower seed oil and its active compound acacetin exerts significant inhibitory effects on UVB-induced MMP-1 expression in HaCaT cells, immortalized human keratinocyte and primary cultured human dermal fibroblasts (HDF) via down regulation of c-Jun and $p90^{RSK}$ phosphorylation. These finding shows that SSO and acacetin could be used as a potential therapeutic anti-wrinkle agent.

II. Materials & methods

2.1. Chemicals

SSO was obtained from the Kerfoot Group (Northallerton, NY) and acacetin was obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified eagle medium (DMEM) was purchased from Hycolne (Long, UT). Fetal bovine serum (FBS), β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO). The MMP-1 antibody was obtained from R&D Systems Inc. (Minneapolis, MN, USA). Antibodies against phosphorylated extracellular-signal regulated kinase 1/2 (ERK1/2) (Thr^{202}/Tyr^{204}), total ERK1/2, total c-Jun N-terminal kinase 1/2 (JNK1/2), phosphorylated-p38 (Thr^{180}/Tyr^{182}), and total p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The other antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA). 3-[4,5-

dimethylatiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) powder was purchased from USB co. (Cleveland, OH). Penicillin/streptomycin was purchased from Invitrogen (Grand Island, NY). Protein assay kits were obtained from Bio-Rad Laboratories (Hercules, CA). 4-(4-Aminophenyl)-1H-indazol-3-ylamine (3-aminoindazole compound) was obtained from MERCK Millipore (Nottingham, UK).

2.2. Cell culture

Primary HDF were isolated from the outgrowth of foreskin obtained from 7 to 30 year old healthy volunteers from Dr. Chung JH laboratory (Seoul National University Hospital, Korea) under the Institutional Review Board at the Seoul National University Hospital and the Seoul National University. HDF were cultured in 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin at 37℃ and 5% CO_2.

Immortalized human keratinocyte HaCaT cells (N.E. Fusenig, Deutsches Krebsforschungszentrum, Heidelberg, Germany) were cultured in same condition with HDF.

2.3. UVB irradiation

UVB irradiation was performed in serum-free media. The spectral peak from the UVB source (Bio-Link crosslinker, VilberLourmat, Cedex 1, France) was set at 312 nm. Primary HDF were exposed to UVB at a dose of 0.02 J/cm^2, and HaCaT cells were exposed to UVB at 0.01 J/cm^2.

2.4. Cell viability

The cell cytotoxicity was measured using the MTT assay. HDF were cultured in the 96 well plates at a density of 2×10^3

cells/well and incubated in DMEM-10% FBS containing penicillin/streptomycin at 37℃ in a 5% CO_2 atmosphere, and HaCaT cells were cultured in the 96well plates at a density of 10×10^5 cells/well and incubated at same condition with HDF. Cells were starved in serum-free DMEM for 24 h. The cells and each sample were incubated for 22 h at 37℃, followed by treatment with MTT solution for 2 h. The medium was removed and formazan crystals were dissolved by the addition of dimethyl sulfoxide (DMSO). The absorbance at 570 nm was then measure using a microplate reader (Molecular Devices, CA) and SSO and acacetin treated and non-treated cells were compared.

2.5. Western blot analysis

HDF and HaCaT cells were cultured for 48 h, and then the cells were incubated in serum-free DMEM for 24 h. After that, the

cells were treated with or without various concentrations of SSO (25, 100 and 400 μg/ml) for 1 h, followed by UVB (0.02 J/cm2 for HDF, 0.01 J/cm^2 for HaCaT cells) irradiation. The media was harvested on ice, and then centrifuged at 18,620 g for 10 min. The protein concentration was measured using a protein assay reagent kits as described by the manufacturer. The proteins were separated electrophoretically using a 10 % SDS-polyacrylamide gel and transferred onto an Immobilon P membrane (MERK Millipore). The membrane was blocked in 5 % fat-free milk for 1 h, and then incubated with the specific primary antibody at 4 ℃ overnight. Protein bands were visualized using a chemiluminescence detection kit (GE healthcare, London, UK) after hybridization with the HRP-conjugated secondary antibody (Life technologies). Western blot data were quantified using the program ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

2.6. Gelatin zymography

Gelatin zymography was performed in 12 % polyacrylamide gel in the presence of gelatin (0.1 % w/v) as a substrate for MMP-2. The protein samples were mixed with loading buffer [10 % SDS, 25 % glycerol, 0.25 M Tris (pH 6.8) and 0.1 % bromophenol blue], and then run on 12 % SDS-PAGE gel without denaturation. Afterward, the gel was washed with renaturing buffer (Life technologies) for 1 h at room temperature and incubated for 24 h at 37 ℃ in developing buffer (Life technologies). After enzyme reaction, the gel was stained with 0.5 % Coomassie brilliant blue in 10 % acetic acid.

2.7. Real-time reverse transcriptase PCR

HDF and HaCaT cells were treated with SSO and acacetin

for 24 h and harvested in RNAiso Plus (Takara Bio, Inc., Shiga, Japan). RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). After reverse transcript with oligo-dT primers using a PrimeScriptTM 1st strand cDNA synthesis Kit (Takara Bio Inc.), Real-time quantitative RT-PCR was performed using IQ SYBR (Bio-Rad Laboratories) and 2 μl of cDNA in triplicate with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. Before PCR amplification, the primers were denatured at 95 ℃ for 3 min. Amplification was made up of 44 cycles at 95 ℃ for 10 sec, 60 ℃ for 30 sec, and 72 °C for 30. PCR was performed by CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). cDNA was probed by the following primer: MMP-1 forward (5'-CCC CAA AAG CGT GTG ACA GTA-3'); MMP-1 reverse (5'-GGT AGA AGG GAT TTG TGC G-3'); GAPDH forward (5'-GAG

TCA ACG GAT TTG GTC GT-3'); GAPDH reverse (5'-TTG ATT TTG GAG GGA TCT CG-3').

2.8. Mitogen-Activated Protein Kinases Profile

The level of phosphorylation of nine mitogen-activated protein kinases (MAPK) and nine other serine/threonine kinases (ERK1, ERK2, JNK1, JNK2, JNK pan, p38α, p38β, p38γ, p38δ, RSK1, RSK2, GSK3α/β, GSK-3β, Akt1, Akt2, Akt3, Akt pan, MSK2, HSP27, and p70 S6 kinase) were determined in the different experimental conditions by commercially available Proteome Profiler antibody array (Human phospho-MAPK Array Kit, RD Systems) (Table 1.), according to the manufacturer instructions. Briefly, 300 μg of cell lysates were diluted in Array Buffer and incubated overnight with the Human Phospho-MAPK Arrays. After binding and removing the unbound material, a cocktail of phospho-

site specific biotinylated antibodies were used to detect phosphorylation via Streptavidin-HRP and chemiluminescence. Phospho-MAPK Array data on developed X-ray film was quantified using the NIH image software analysis.

2.9. Statistical analysis

If it is necessary, the data were expressed as means ± standard deviation (S.D.). The Student's t-test was used for statistical comparisons. $p < 0.05$ and $p < 0.01$ were used as criterion for statistical significance.

III. Results

3.1. SSO inhibits UVB-induced MMP-1 protein expression in HaCaT cells and HDF.

To investigate the anti-wrinkle effect of SSO, I evaluated the inhibitory effects on UVB-induced MMP-1 protein expression in HaCaT cells and HDF. SSO significantly suppressed UVB-induced MMP-1 expression in HaCaT cells (Fig. 1A and B), and HDF (Fig. 1C and D) at the concentration which did not show cytotoxicity (Fig. 1E). This result suggests that SSO inhibits UVB-induced MMP-1 protein expression in HaCaT cells and HDF.

Figure 1

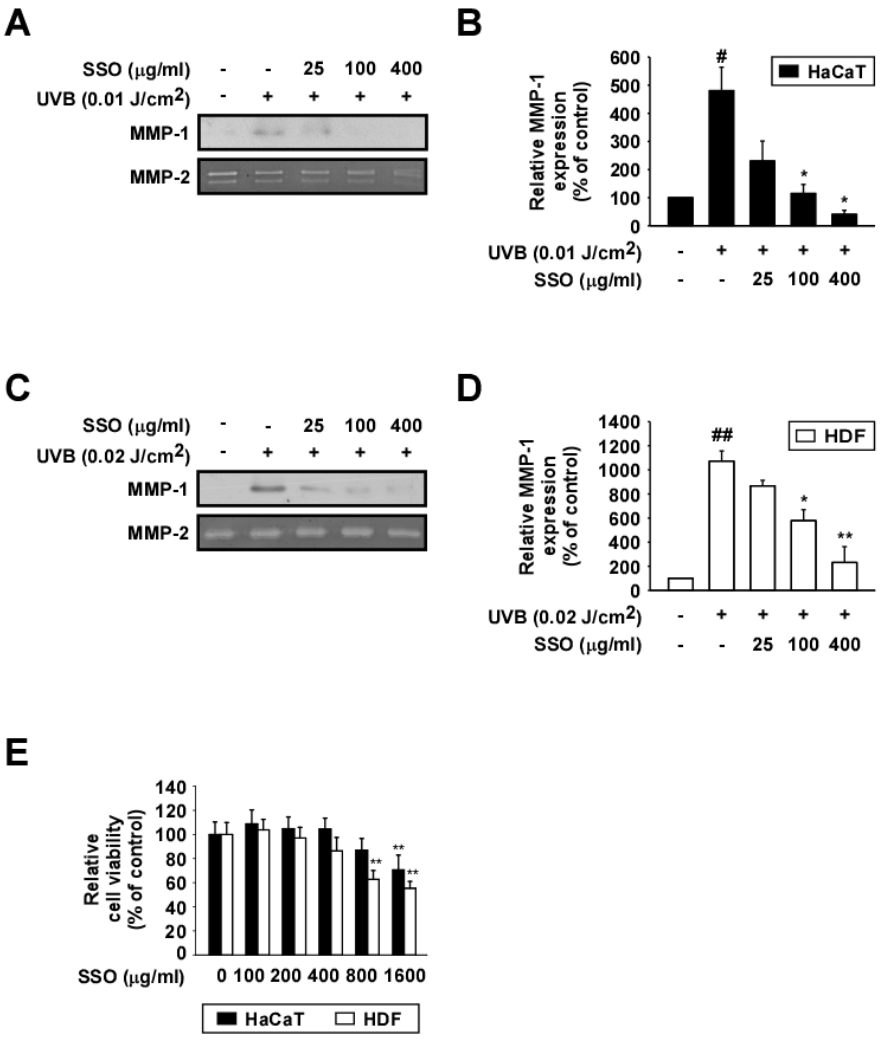
A
SSO (μg/ml) - - 25 100 400
UVB (0.01 J/cm2) - + + + +
MMP-1
MMP-2
B
Relative MMP-1 expression (% of control)
600
500
400
300
200
100
0
#
*
*
HaCaT
UVB (0.01 J/cm2) - + + + +
SSO (μg/ml) - - 25 100 400
C
SSO (μg/ml) - - 25 100 400
UVB (0.02 J/cm2) - + + + +
MMP-1
MMP-2
D
Relative MMP-1 expression (% of control)
1400
1200
1000
800
600
400
200
0
##
*
**
HDF
UVB (0.02 J/cm2) - + + + +
SSO (μg/ml) - - 25 100 400
E
Relative cell viability (% of control)
140
120
100
80
60
40
20
0
**
**
**
SSO (μg/ml) 0 100 200 400 800 1600
HaCaT
HDF

Figure 1. Effect of SSO on UVB-induced MMP-1 protein expression in HaCaT cells and HDF. *A.* Expression of MMP-1 was determined by Western blot. MMP-2 was used as a loading control. Cells were pretreated with SSO at the indicated concentrations for 1 h, and then further treated with 0.01 J/cm^2 UVB in HaCaT cells for 48 h at 37℃. *B.* MMP-1 protein expression data of HaCaT cells were quantified using the NIH image software analysis. Data (n = 3) represent the mean ± SD. *C.* Expression of MMP-1 was determined by Western blot and MMP-2 was also used as a loading control. Cells were pretreated with SSO at the indicated concentrations for 1 h, and then further treated with 0.02 J/cm^2 UVB in HDF for 48 h at 37℃. *D.* MMP-1 protein expression data of HDF were quantified using the NIH image software analysis. Data (n = 3) represent the mean ± SD. *E.* Cell viability of SSO was measured using the MTT assay as described in the Materials and Methods. Data (n = 5) are shown as the

means ± SD. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, relative to control cells. $^{*}p < 0.05$ and $^{**}p < 0.01$, relative to UVB-induced cells.

3.2. Acacetin effectively attenuates UVB-induced MMP-1 protein expression in HaCaT cells and HDF.

To verify anti-aging effects of acacetin, I tested the effect of acacetin on UVB-induced MMP-1 protein expression in HaCaT cells and HDF. Acacetin effectively attenuated UVB-induced MMP-1 protein expression in HaCaT cells (Fig. 2B and C) and HDF (Fig. 2D and E). Acacetin did not show cytotoxicity in both cells up to 10 μM (Fig. 2F).

Figure 2

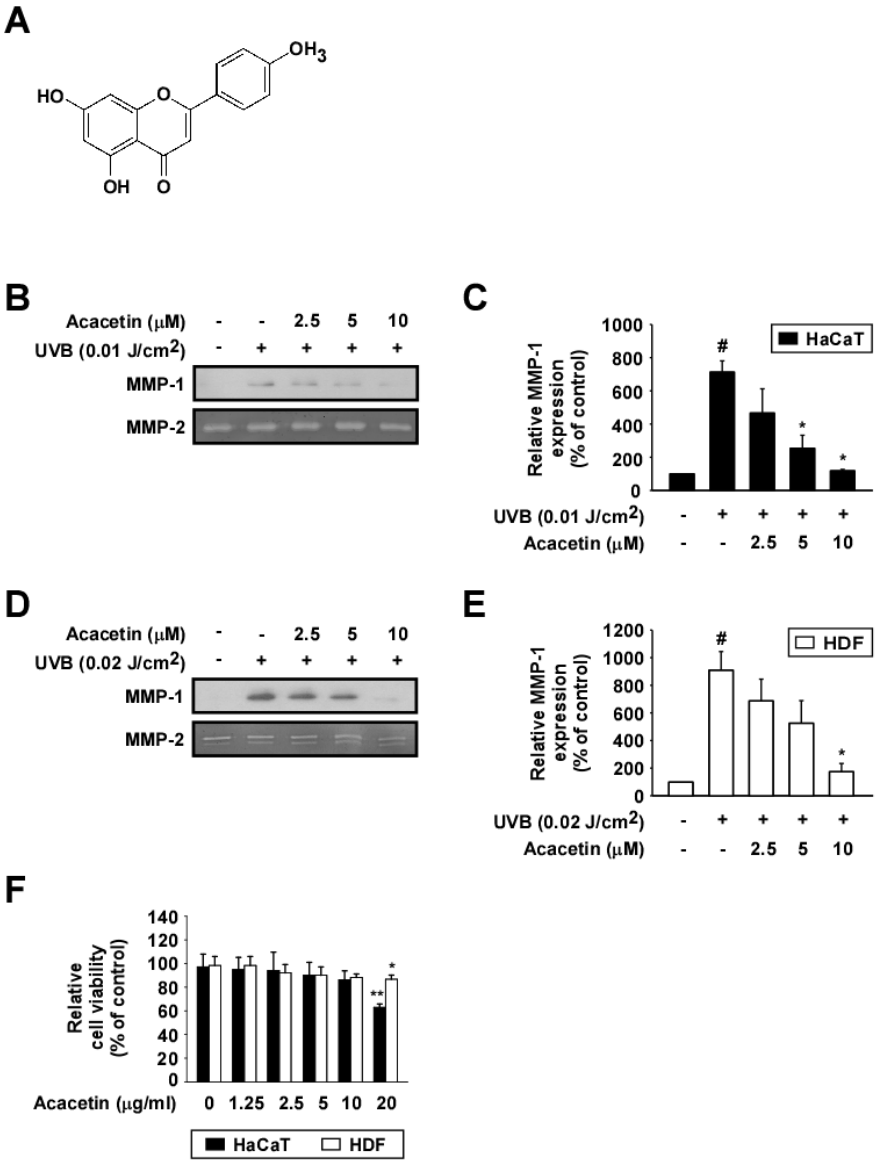
A
HO
O
OH3
OH
O
B
Acacetin (μM) - - 2.5 5 10
UVB (0.01 J/cm2) - + + + +
MMP-1
MMP-2
C
Relative MMP-1 expression (% of control)
1000
800
600
400
200
0
#
*
*
HaCaT
UVB (0.01 J/cm2) - + + + +
Acacetin (μM) - - 2.5 5 10
D
Acacetin (μM) - - 2.5 5 10
UVB (0.02 J/cm2) - + + + +
MMP-1
MMP-2
E
Relative MMP-1 expression (% of control)
1200
1000
800
600
400
200
0
#
*
HDF
UVB (0.02 J/cm2) - + + + +
Acacetin (μM) - - 2.5 5 10
F
Relative cell viability (% of control)
140
120
100
80
60
40
20
0
**
*
Acacetin (μg/ml) 0 1.25 2.5 5 10 20
HaCaT
HDF

Figure 2. Effects of acacetin on UVB-induced MMP-1 protein expression in HaCaT cells and HDF. *A.* Chemical structure of acacetin. *B.* Expression of MMP-1 was determined by Western blot. MMP-2 was used as a loading control. Cells were pretreated with acacetin at the indicated concentrations for 1 h, and then further treated with 0.01 J/cm^2 UVB in HaCaT cells for 48 h at 37℃. *C.* MMP-1 protein expression data of HaCaT cells were quantified using the NIH image software analysis. Data (n = 3) represent the mean ± SD. *D.* Expression of MMP-1 on HDF was determined by Western blot and MMP-2 was also used as a loading control. Cells were pretreated with acacetin at the indicated concentrations for 1 h, and then further treated with 0.02 J/cm^2 UVB in HDF for 48 h at 37℃. *E.* MMP-1 protein expression data of HDF were quantified using the NIH image software analysis. Data (n = 3) represent the mean ± SD. *F.* Cell viability of acacetin was measured using the MTT assay. Cells

were starved in serum-free DMEM for 24 h and cells and acacetin were incubated for 22 h at 37℃, followed by treatment with MTT solution for 2 h. Data (n = 5) represent the mean ± SD. $^{\#}p < 0.05$, relative to control cells. $^{*}p < 0.05$ and $^{**}p < 0.01$, relative to UVB-induced cells.

3.3. SSO and acacetin inhibit UVB-induced MMP-1 mRNA expression in HaCaT cells and HDF.

Effects of SSO and acacetin on UVB-induced MMP-1 mRNA expression was examined by real-time PCR. The results indicate that SSO significantly suppressed UVB-induced MMP-1 mRNA expression in HaCaT cells and HDF in a dose-dependent manner (Fig. 3A and B), and acacetin also has inhibition effects in both two cells in dose-dependent manner (Fig. 3C and D).

Figure 3

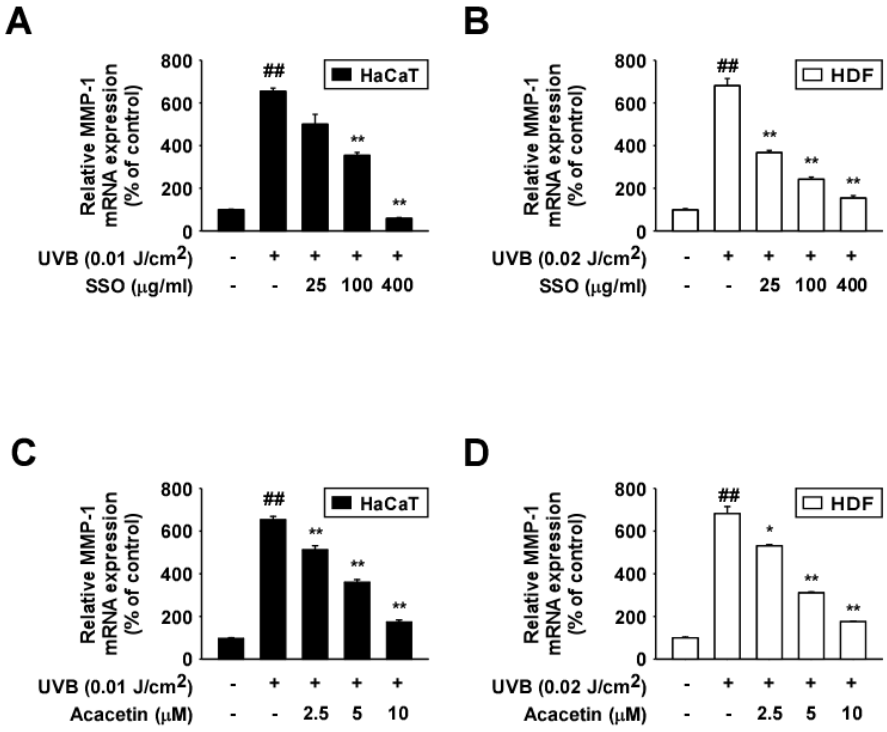
A
Relative MMP-1 mRNA expression (% of control)
800
600
400
200
0
##
**
**
**
HaCaT
UVB (0.01 J/cm2) - + + + +
SSO (μg/ml) - - 25 100 400
B
Relative MMP-1 mRNA expression (% of control)
800
600
400
200
0
##
**
**
**
HDF
UVB (0.02 J/cm2) - + + + +
SSO (μg/ml) - - 25 100 400
C
Relative MMP-1 mRNA expression (% of control)
800
600
400
200
0
##
**
**
**
HaCaT
UVB (0.01 J/cm2) - + + + +
Acacetin (μM) - - 2.5 5 10
D
Relative MMP-1 mRNA expression (% of control)
800
600
400
200
0
##
*
**
**
HDF
UVB (0.02 J/cm2) - + + + +
Acacetin (μM) - - 2.5 5 10

Figure 3. Effect of SSO and acacetin on UVB-induced MMP-1 gene transcription in HaCaT cells and HDF. *A.* MMP-1 mRNA level was analyzed by real-time quantitative PCR. Cells were pretreated with SSO at the indicated concentrations for 1h, and then further treated with 0.01 J/cm^2 UVB in HaCaT cells and *B.* in 0.02 J/cm^2 UVB in HDF for 48h at 37℃. Data (n = 3) represent the mean ± SD. *C.* MMP-1 mRNA level was analyzed by real-time quantitative PCR. Cells were pretreated with 0.01 J/cm^2 UVB in HaCaT cells and *D.* in 0.02 J/cm^2 UVB in HDF for 48h at 37℃. Data (n = 3) represent the mean ± SD. $^{\#\#}p < 0.01$, relative to control cells. $^{*}p < 0.05$ and $^{**}p < 0.01$, relative to UVB-induced cells.

3.4. Acacetin decreases MMP-1 transcription via attenuating phosphorylation of c-Jun and p90^{RSK} signal transductions.

Base on the previous study, MAPK signaling pathway plays an important role in regulating MMP-1 expression [37]. Thus, I used a human phospho-MAPK array to monitor changes in UVB-induced phosphorylation states in response to SSO and acacetin in HDF using MAPK profiling assay. This array included major MAPK families (ERK, p38 and JNK) as well as intracellular kinases, such as Akt, MKK, p70^{S6K} and so on, which were important in signal transduction and regulating MMP-1 expression [38]. The result indicates that there are no significant inhibition of UVB-induced phosphorylation of kinase (Fig. 4A and B). After the examination the downstream kinase of MAPK, the results demonstrate that acacetin decreased UVB-induced phosphorylation of c-Jun and p90^{RSK} (Fig. 5A). Overall, I found that acacetin, active compound of SSO effectively suppressed

UVB-induced MMP-1 expression by inhibiting the UVB-induced phosphorylation of c-Jun and $p90^{RSK}$ in HDF.

Figure 4

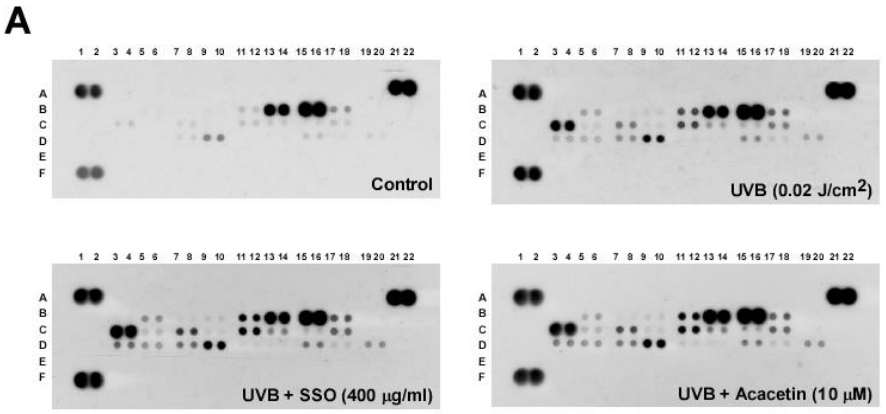
A
Control
UVB (0.02 J/cm2)
UVB + SSO (400 μg/ml)
UVB + Acacetin (10 μM)
B
Density (% of control)
3000
2500
2000
1500
1000
500
0
Akt1
Akt2
Akt3
Akt pan
CREB
ERK1
ERK2
GSK-3α/β
HSP27
JNK1
JNK2
JNK3
JNK pan
MKK3
MKK6
MSK2
p38α
p38β
p38δ
p38γ
p70S6K
p53
RSK1
RSK2
TOR

Figure 4. Effect of safflower seed oil and acacetin on UVB-induced phosphorylation of MAPK signaling. *A.* The level of phosphorylation of MAPK and other serine/threonine kinases were determined in the different experimental conditions by commercially available Proteome Profiler antibody array. HDF were treated with or without 0.02 J/cm^2 of UVB. SSO (400 μg/ml) and acacetin (10 μM) was treated with 0.02 J/cm^2 of UVB for 30 min. After treatment, HDF were washed and lysed. Twenty-six different intracellular phosphorylated MAPK and other serine/threonine kinases in total cellular lysate were assessed semi-quantitatively using an antibody based Human Phospho-MAPK Array Kit. Positive and negative controls were designated at (A1, A2, A21, A22, F1, F2) and (E19, 20), respectively. The format of the antibodies on the array are detailed in Table 1. *B.* Phospho-MAPK Array data on developed X-ray film was quantified using the NIH image software analysis.

Figure 5

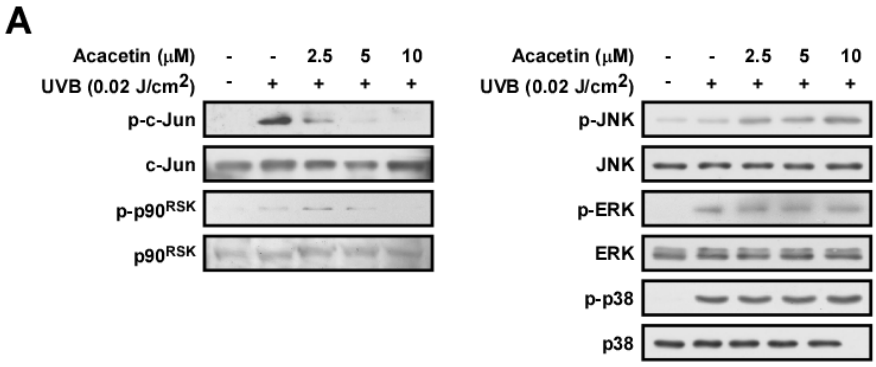
A
Acacetin (μM) - - 2.5 5 10
UVB (0.02 J/cm2) - + + + +
p-c-Jun
c-Jun
p-p90RSK
p90RSK
Acacetin (μM) - - 2.5 5 10
UVB (0.02 J/cm2) - + + + +
p-JNK
JNK
p-ERK
ERK
p-p38
p38

Figure 5. Effects of acacetin on UVB-induced signaling pathways in HDF. *A.* Acacetin dose-dependently inhibits UVB-induced phosphorylation of c-Jun and p-90^{RSK}. The phosphorylation of p-JNK, p-ERK, p-38 were not suppressed by acacetin. Each of the phosphorylation and total protein levels were determined with western blot analysis using specific antibodies described in the Materials and Methods.

Table 1

Table 1. The format of antibodies on the Human Phospho-MAPK membrane array

Coordinate	Total/control	Alternate nomenclature	Phosphorylation site detected
A1, A2	Positive control	-	-
A21, A22	Positive control	-	-
B3, B4	Akt1	PKBα, RACα	S473
B5, B6	Akt2	PKBβ, RACβ	S474
B7, B8	Akt3	PKBγ, RACγ	S472
B9, B10	Akt pan	-	S473, S474, S472
B11, B12	CREB	-	S133
B13, B14	ERK1	MAPK3, p44 MAPK	T202/Y204
B15, B16	ERK2	MAPK1, p42 MAPK	T185/Y187
B17, B18	GSK-3α/β	GSK3A/GSK3B	S21/S9
B19, B20	GSK-3β	GSK3B	S9
C3, C4	HSP27	HSPB1, SRP27	S78/S82
C5, C6	JNK1	MAPK8, SAPK1γ	T183/Y185

Table 1. continue

Coordinate	Total/control	Alternate nomenclature	Phosphorylation site detected
C7, C8	JNK2	MAPK9, SAPK1α	T183/Y185
C9, C10	JNK3	MAPK10, SAPK1β	T221/Y223
C11, C12	JNK pan	-	T183/Y185, T221/Y223
C13, C14	MKK3	MEK3, MAP2K3	S218/T222
C15, C16	MKK6	MEK6, MAP2K6	S207/T211
C17, C18	MKK2	RSKβ, RPS6KA4	S360
D3, D4	p38α	MAPK14, SAPK2A, CSBP1	T180/Y182
D5, D6	p38β	MAPK11, SAPK2B, p38-2	T180/Y182
D7, D8	p38δ	MAPK13, SAPK4	T180/Y182
D9, D10	p38γ	MAPK12, SAPK3, ERK6	T183/Y185
D11, D12	p53	-	S46
D13, D14	p70 S6 Kinase	S6K1, p70α, RPS6KB1	T421/S424
D15, D16	RSK1	MAPKAPK1α, RPS6KA1	S380
D17, D18	RSK2	ISPK-1, RPS6KA3	S386
D19, D20	TOR	-	S2448
E19, E20	PBS	Control (-)	-
F1, F2	Positive control	-	-

IV. Discussion

Recent studies indicate that sun exposure is a major environmental factor of aggravation of skin aging. Skin aging is a process of senescence and is commonly related to wrinkling, sagging and laxity [2, 39]. It is divided into two types; extrinsic and intrinsic aging. Extrinsic aging is generally correlated with photoaging since it is caused by intense and chronic UV light exposure and is characterized by severe wrinkling and pigmental changes while intrinsic aging is characterized by smooth, dry, pale and finely wrinkled skin.

Photoaging is caused by degradation of collagen in dermis. Because of skin aging, collagenases called MMPs are excessively expressed. MMPs are responsible for the degradation or synthesis inhibition of collagenous extracellular matrix (ECM) in connective

tissues [13]. Various MMPs including MMP-1 (collagenase), MMP-3 (stromelysine-1), and MMP-9 (gelatinase B) are increased in the human skin or dermal fibroblasts when exposed to UVB, but not MMP-2 (gelatinase A) [40, 41]. MMP-1 initiates cleavage of fibrillar collagen to make triple helix coil into a single site [42]. Once collagen febrile cleaved by MMP-1, it can be further degraded by elevated levels of MMP-3 and MMP-9. Therefore, MMP-1 plays a crucial role for a main enzyme in deteriorating wrinkle formation and ECM degradation [43]. Accordingly, the inhibition of UVB-induced MMP-1 overexpression represents an effective strategy to prevent photoaging.

Safflower (*Carthamus tinctorius L.*) has been cultivated for a long time to get vegetable oil from its seed. It is widely grown in eastern and western Asia. SSO is rich in linoleic acid and oleic acid which are unsaturated fatty acids [24]. Many previous studies

revealed that SSO lower blood cholesterol level and prevent degradation of bone loss [30, 31]. There are a few study conducted for reveal the polyphenol from SSO, so it is reveal that there are a lot of polyphenols in safflower seed, leaf and flower. Acacetin (5,7-dihydroxy-4′-methoxyflavone) is a major flavonoids isolated from safflower seeds, plants and leaves [32], and it is able to exert anti-peroxidative, anti-inflammatory, anti-plasmodial and anti-proliferative activities [33, 34]. There are a lot of studies about biological effects of acacetin, however anti-wrinkle effects has not been reported [35, 36]. Therefore, I focused on the anti-wrinkle effects of SSO and its active compound acacetin, and then monitored changes in UVB-induced phosphorylation using MAPK profiling assay.

In this study, SSO and acacetin showed anti-wrinkle effects via modulating major human collagenase, MMP-1. Its mRNA level

also inhibited dose dependent in immortalized human keratinocyte HaCaT and primary cultured HDF (Fig. 1, 2 and 3).

Upon UV irradiation, MAP kinase signal transduction pathway is activated, which mediates downstream cellular response [44]. Since previous studies have demonstrated that MMP-1 expression is regulated by MAPK activation, the profiling assay of SSO and acacetin on UVB-induced phosphorylation of MAPK was investigated. Although there is no significant down-regulation of UVB-induced phosphorylation of MAPK in profiling assay (Fig. 4), it is possible to regulate the protein located near the membrane-binding protein which regulates MMP-1 mRNA. Finally, I confirmed its inhibitory effects on downstream signal transduction cascade by western blot analysis.

The results showed that UVB-induced phosphorylation of c-Jun and $p90^{RSK}$ were decreased in acacetin treated cells, and these

observations suggest that acacetin attenuates UVB-induced MMP-1 expression by inhibiting c-Jun and $p90^{RSK}$ signaling pathway (Fig. 5)

In summary, these results indicate that SSO and its active compound acacetin protect against UVB-induced skin photoaging in HaCaT cells and HDF. I discovered that acacetin prevents UVB-induced MMP-1 expression via attenuating phosphorylation of c-Jun and $p90^{RSK}$ signal transductions. Therefore, SSO and acacetin have a potential to be utilized as alternative anti-wrinkle agents.

V. References

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VI. 국문초록

잇꽃씨유는 올레익산과 리놀레익산과 같은 불포화 지방산을 많이 포함하고 있으며, 이러한 불포화 지방산은 손상된 피부 장벽을 보호하고 건조한 피부나 머리카락에 도움을 준다고 보고된 바 있다. 피부의 주름을 감소시키는 다양한 항노화 소재들이 많이 밝혀져 있지만, 피부 보습에 도움을 주는 동시에 주름 형성을 억제하는 잇꽃씨유의 항노화 효능을 밝힌 연구는 보고된 바 없다. 본 연구에서는 잇꽃씨유이 노화에 의한 피부 주름을 억제하는 효능을 밝혀, 새로운 항노화 소재로서 이용될 수 있음을 제시하였다.

피부 주름은 지속적인 자외선 노출에 의해 유도되며 이는 피부 속 콜라겐을 분해하는 콜라겐 분해 효소 (Matrix metalloproteinase, MMP-1)의 발현 증가에 의해 이루어진다. 잇꽃씨유는 피부 각질세포와 진피세포에서 자외선에 의해 유도된 MMP-1 단백질의 발현을 농도 의존적으로

억제하였으며, 이는 MMP-1 mRNA의 발현이 억제됨으로써 조절되는 것을 확인하였다. 또한 잇꽃씨유의 핵심성분인 아카세틴 역시 두 가지 세포 모델에서 자외선에 의해 유도된 MMP-1 단백질과 mRNA 발현을 억제하는 것을 확인하였다. Western blot과 Proteome profiler antibody array를 통해 전사인자를 조절한다고 알려진 MAPKs 신호전달 체계를 확인한 결과, 아카세틴이 자외선에 의해 유도된 c-Jun 단백질과 p-90^{RSK} 단백질의 인산화를 농도 의존적으로 억제하는 것을 확인하였다.

본 연구 결과를 통해 잇꽃씨유와 그 핵심 성분인 아카세틴은 자외선에 의해 유도된 피부 노화를 효과적으로 억제함을 밝혔으며, 잇꽃씨유가 피부 주름을 억제하는 신규한 항노화 소재로서 사용될 수 있는 가능성을 제시하였다.