A Thesis for the Degree of Master of Science

Brown pine leaf extract and its active component *trans*-communic acid inhibit ultraviolet B induced matrix metalloproteinase-1 expression in 2D and 3D skin cell culture system

갈색솔잎추출물과 유효성분 *trans*-communic acid의 인간각화세포 및 생체피부모방모델에서 자외선에 의한 MMP-1 발현 저해효과 및 작용기전 규명

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서울대학교 대학원 농생명공학부

허 원 범

허원범의 석사 학위 논문을 인준함

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Abstract

Pine (*Pinus densiflora*), Korean national tree, has grown in all parts of Korea peninsula. Every two year, Pine leaf changes its color to brown and falls. Even though this 'brown pine leaf' is widely spread, we have not paid much attention to its value. In this study, brown pine leaf is suggested as an anti-photoaging agent to our skin. Brown pine leaf extract (BPLE) inhibits UVB-induced matrix metalloproteinase-1 (MMP-1) expression more than pine leaf extract (PLE) in human keratinocyte and human skin equivalent model. HPLC analysis shows that the quantity of *trans*-communic acid (TCA) and dehydroabietic acid (DAA) increased by the pine leaf color change from green to brown. BPLE and TCA are able to reduce UVB-induced MMP-1 mRNA expression and activator protein-1 (AP-1) transactivation. However, UVB-induced mitogen activated protein kinase (MAPK), which is known to regulate AP-1 transactivation, was not affected by BPLE and TCA. Compared to UVB only treated group, BPLE and TCA treated groups attenuate collagen degradation and the MMP-1 protein level. In sum, both BPLE and its

active component TCA exhibit possible chemopreventive activity against UVB-induced skin aging.

Keywords: Brown pine leaf extract; *trans*-communic acid; Ultraviolet B; MMP-1; Skin equivalent

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Contents

I. Introduction

Human skin works as an interface between environment and our body [1]. Chronic ultraviolet (UV) exposure causes a lot of detrimental physiological disorder in our skin such as epidermal inflammation, hyperpigmentation, immunosuppression and photoaging [2-5]. UV could be divided into UVA (320-400 nm), UVB (280-320 nm), UVC (200-280 nm)[6]. UVA and UVB are able to pass ozone layer and reach to human skin. In this UV UVA consists 95% and UVB takes up 5 %. Especially UVB plays major role in skin aging because it has higher energy than of UVA [7].

Photoaging which is an extrinsic aging is characterized to pigmentary changes and severe wrinkle formation [8]. Severe wrinkle is formed by collagen breakdown from UV-induced collagenase. It is also related to extracellular matrix breakdown, which causes detrimental effect to connective tissues in various pathological situations [9-12]. Matrixmetalloproteinase-1 (MMP-1), one of the MMP collagenase family such as MMP-1, MMP-2, MMP-9, MMP-13, etc [13]is a critical enzyme for type-1 collagen and type-3 collagen degradation in human skin [4, 7].

Preventing unbalanced collagen degradation via inhibition of MMP-1 expression can be one of the excellent strategies for anti-photoaging.

UVB irradiation activates cell surface receptors including EGFR. This leads to propagation of intracellular signaling such as mitogen-activated protein kinases (MAPKs), PI3K/AKT, Jak/STAT, and protein kinase-C [14-17]. AP-1 is a transcription factor that plays a crucial role in transcription of UVB-induced target gene including MMP-1. AP-1 family is composed of Jun proteins (c-Jun, v-Jun, JunB, JunD) and Fos proteins (c-Fos, v-Fos, FosB, Fra-1, Fra-2), or activating transcription factors (ATF2, ATF3/LRF1, B-ATF) and basic region leucine zipper (bZIP) homodimerize or heterodimerize [18-20].

Pine (*Pinus densiflora*) is affilled with the Pinaceae family has been cultivated in China, Russia, Japan, and Korea [21]. In Korea, approximately 65 % of land is a forest and pine occupies about 87 % of coniferous forests [22]. Pine leaf stays for 2 years and loses its vigor and color changes to brown [23]. Although this ‘brown pine leaf’ is widely spread in forest and park, it has not been studied as a functional material.

In this paper, anti-photoaging effect of brown pine leaf extract

(BPLE) and its active component *trans*-communic acid (TCA) is studied. BPLE inhibits UVB-induced MMP-1 expression more than that of PLE. BPLE and TCA both inhibit UVB-induced collagen degradation through inhibition of MMP-1 in human skin equivalent model.

II. Materials & methods

2.1. Chemicals and reagents

Dulbecco's modified eagle medium (DMEM) was purchased from Hycolne (Long, UT). Fetal bovine serum (FBS) and β-actin antibody were bought from Sigma-Aldrich (St.Louis, MO). The MMP-1 antibody was obtained from R&D systems (R&D systems, MN). Antibodies against phosphorylated extracellular-signal regulated kinase 1/2 (ERK1/2) (Thr202/Tyr204), total ERK1/2, total c-Jun N-terminal kinase 1/2 (JNK1/2), phosphorylated-p38 (Thr180/Tyr182), and total p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated JNK1/2 (Thr183/Tyr185), phosphorylated p90RSK (Thr359/Ser363) and total p90RSK were purchased from Cell signaling (Cell signaling. 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).

2.2. Preparation of plant extracts

Green and brown color leaves of *Pinus densiflora* Siebold & Zucc (Pine leaf) were collected in Amorepacific botanical garden. Dried green pine leaves and brown pine leaves were ground and extracted with ten volumes of 80 % ethanol for 2 days at room temperature. The extracts were obtained after filtration and solvent evaporation.

2.3. UVB irradiation

UVB irradiation was conducted with serum-free media. The spectral peak of UVB (Bio-Link crosslinker, VilberLourmat, Cedex 1, France) was set at 312 nm. HaCaT cell was exposed to UVB at 0.01 J/cm^2.

2.4. Cell culture

HaCaT cells were cultured in 10 % (v/v) FBS added Dulbecco's modified Eagle's medium (DMEM) with 1 μg/ml penicillin/streptomycin at 37 °C and 5 % CO_2 atmosphere. For luciferase assay, HaCaT cells

were stably transfected with AP-1 luciferase reporter plasmid. This transfected HaCaT cells were cultured in 10 % (v/v) FBS added Dulbecco's modified Eagle's medium (DMEM) with 1 μg/ml puromysin.

2.5. Human Skin equivalent preparation

Human skin equivalent (Neoderm®-ED) was purchased from Tegoscience (Seoul, Korea). The human skin equivalent were treated with BPLE (5, 10 μg/ml), TCA (5, 10 μM) for one hour after 2 weeks of air-lift. Skin equivalent was irradiated with UVB at 0.05 J/cm^2 twice a day for 8 days. Medium was changed every 2 days during 8 days. Skin equivalent was incubated at 37 °C and 5 % CO_2 atmosphere.

2.6. Isolation and purification single compound

Brown pine leaves (100 g) were ground and extraction was conducted with ten volume of 80 % ethanol for 2 days at room temperature. BPLE (11.4 g) was sequentially fractionated with hexane (HA), chloroform ($CHCl_3$), butyl alcohol (BuOH), and water. The HA

fraction (2.93) applied to a silica gel column (60, 70-230 mesh, Merck & Co., Whitehouse Station, NJ, USA) and was eluted with chloroform and methanol solution (10:0.7 v/v) to obtain five fractions (Fractions I-V). Fraction II was subjected to Rp-18 column chromatography (LiChroprep® RP-18 25-40 μm, Merck & Co.) and was eluted with 70 % methanol, which eventually yielded compound II-C (826 mg). By referring to literature [24] and comparing compound II-C spectral data obtained from several methods such as ^{13}C nuclear magnetic resonance (NMR), ^{1}H-NMR, and electron-ionization mass spectrometry (EI-MS), the chemical structure of compound II-C is found to be TCA or 1,4a-Dimethyl-6-methylene-5-(3-methyl-penta-2,4-dienyl)-decahydro-naphthalene-1-carboxylic acid.

2.7. Cell viability

Cell viability was measured by MTT assay. HaCaT cells were cultured in96 well plates at a density of 10×10^5 cells/well and were incubated in DMEM-10% FBS containing penicillin/streptomycin at 4 °C in 5 % CO_2 atmosphere. After reaching 80% of cell confluence, HaCaT

cells were starved in serum-free DMEM for 24 h. The cells treated with different dosage samples were incubated for 22 h at 37 °C. After incubation, MTT solution treatment was performed for 2 h. The medium was removed and the remaining formazan crystals in the cells were dissolved by addition of dimethyl sulfoxide (DMSO). Microplate reader (Molecular Devices, CA) was used to measure the color density of cells at 570nm.

2.8. Luciferase reporter gene assay

HaCaT cells, stably transfected with AP-1 luciferase plasmid, were cultured in 96 well plates at a density of 10×10^5 cells/well. After incubation in DMEM-10 % FBS containing penicillin/streptomycin at 4 °C in 5 % CO_2 atmosphere, the cells were starved in serum-free DMEM for 24 h. Then the cells were treated with various concentration of samples for 1 h followed by UVB (0.01 J/cm^2) irradiation. After 12 hours incubation, the cells were treated with lysis buffer [0.1 mM potassium phosphate buffer (pH 7.8), 2 mM ethylene diamine tetra acetic acid (EDTA), 1 % Triton X-100, 1 mM dithiothreitol (DTT)] and then

their luciferase activities were measured by luminometer (Luminoskan Ascent; Thermo Electron, Helsinki, Finland)

2.9. Real time PCR (RT-PCR)

HaCaT cells (10×10^5 cells in a 6 well dish) were treated with BPLE and TCA for 15 h and harvested in RNAiso Plus (Takara Bio, Inc., Shiga, Japan). After RT with oligo-dTprimers using PrimeScriptTM 1st strand cDNA synthesis Kit (Takara Bio, Inc.), the cDNA was probed by the following primer (Bioneer, Daejeon, Korea): MMP-1 forward 5'-ATT CTA CTG ATA TCG GGG CTT TGA-3', MMP-1 reverse 5'-ATG TCC TTG GGG TAT CCC TGT AG-3' (409 bp); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'-GAG TCA ACG GAT TTG GTC GT-3', GAPDH reverse 5'-TTG ATT TTG GAG GGA TCT CG-3'(517 bp). Before PCR amplification, the primers were denatured at 94 °C for 5 min. Amplification was consisted of 22 cycles: denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min followed by final 5 min extension at 72 °C. PCR was performed by Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA).

RT-PCR reaction was performed by CFX96 real-time PCR detection system (Vio-Rad, Hercules, CA). cDNA was amplified in a presence of iQ^{TM} SYBR®Green Supermix (Bio-Rad). To control variations of mRNA concentration, all results were normalized to the housekeeping gene, GAPDH. Relative quantitation was performed by comparative ΔΔCt method followed by manufacturer's instructions.

2.10. Matrix metalloproteinase-1 contents (Enzyme linked immunosorbent assay, ELISA)

Cultured HaCaT cells were starved in serum free-DMEM for 24 h to exclude any potential FBS activated cell signals. After starvation, the cells were treated with various concentration of samples for 1 h, followed by UVB (0.01 J/cm^2) irradiation. Conditioned media was collected after 48 h of incubation. The samples were measured by the DuoSet human total MMP-1 ELISA kit (R&D system Inc.) as described in manufacturer's protocols.

2.11. Western blot assay

Cultured HaCaT cells with 10% FBS added DMEM is replaced with serum free-DMEM for 24 h to lower the growth signals stimulated by any potential FBS. After starvation, the cells were treated with various concentration of samples for 1 h, followed by UVB (0.01 J/cm^2) irradiation. The cells were scraped with lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 1% Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 % glycerol and protease inhibitor cocktail tablet] on the ice for 20 min, and then centrifuged at 18,620×g for 10 min. The protein concentration were measured by a dye-binding protein assay kit (Bio-Rad Laboratories) as described by the manufacturer. , The proteins were separated electrophoretically in 10 % SDS-polyacrylamide gel and transferred to Immobilon P membrane (Millipore corporation, USA). The membrane was blocked with 5 % fat-free milk for 1 h and then incubated with the specific primary antibody at 4 °C overnight. Protein bands were visualized by chemiluminescence detection kit (GE healthcare, NJ) after hybridization with HRP-conjugated secondary antibody (Invitrogen, USA).

2.12. Zymography

Zymography was used to determine the activity of secreted MMP-2. Zymography was performed in 10 % polyacrylamide gel in the presence of gelatin (0.5 mg/ml) as a substrate for MMP-2. The samples were suspended in loading buffer [10 % SDS, 25 % glycerol, 0.25 M Tris (pH 6.8) and 0.1 % bromophenol blue], and then run on 10 % SDS-PAGE gel without denaturation. After electrophoresis, the gel was washed with renaturing buffer (Invitrogen, USA) at room temperature for 30 min and then incubated in developing buffer (Invitrogen, USA)for 24 h at 37 °C. The gel was then stained with 0.5 % Coomassie brilliant blue.

2.13. Immunohistochemistry

Skin sections from human skin equivalent were prepared for immunohistochemical staining of MMP-1 expression. Sections (5 mm thick) of 10 % neutral formalin solution-fixed paraffin-embedded tissues were cut on silane-coated glass slides. And then they were deparaffinized for three times with xylene, and dehydrated through a graded alcohol bath. The deparaffinized sections were incubated in citric acid buffer (pH

6.0) and boiled for antigen retrieval. To prevent nonspecific staining, each section was treated with 3 % hydrogen peroxide for 10 min and a blocking solution containing 1 % bovine serum albumin (BSA) for 30 min. For the detection of MMP-1 target, the slides were incubated overnight with an affinity-purified primary antibody at 4 °C in 1 % BSA then developed by an anti-rabbit or anti-mouse Histostain Plus Kit (Zymed Laboratories Inc., South San Francisco, CA). Peroxidase binding sites were detected by staining with 3,3' – diaminobenzidinetetrahydrochloride (Sigma-Aldrich). Mayer's hematoxylin was applied as a counterstain (Sigma-Aldrich).

2.14. Masson's trichrome staining

Skin sections from human skin equivalent were prepared for Masson's trichrome staining for collagen. Sections (5 mm thick) of 10 % neutral formalin solution-fixed paraffin-embedded tissues were cut on silane-coated glass slides and then deparaffinized three times with xylene and dehydrated through a graded alcohol bath. The deparaffinized sections were stained with Hematoxylin for 5 min. And then, slides were

washed and stained in biebrich scarlet and acid fuchsin. Next, the slides were placed in phosphomolybdic-phosphotungstic acid for 10 min and aniline blue for 5 min to stain collagen. Slides were then washed and incubated in 1 % acetic acid for 15 min. Lastly, they were dehydrate and wash.

2.15. 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 the criterion for statistical significance.

III. Results

3.1. BPLE has stronger inhibitory effects against UVB-induced MMP-1 expression than that of PLE in human keratinocyte

To determine anti-aging effects of BPLE, BPLE and PLE were tested in human keratinocyte for their inhibitory effect against UVB-induced MMP-1 expression within the range of concentration showoff no cytotoxicity (Fig. 1B). Results indicated that BPLE has stronger inhibitory effects against UVB induced MMP-1 expression than that of PLE in a dose dependent manner (Fig. 1A).

Figure 1

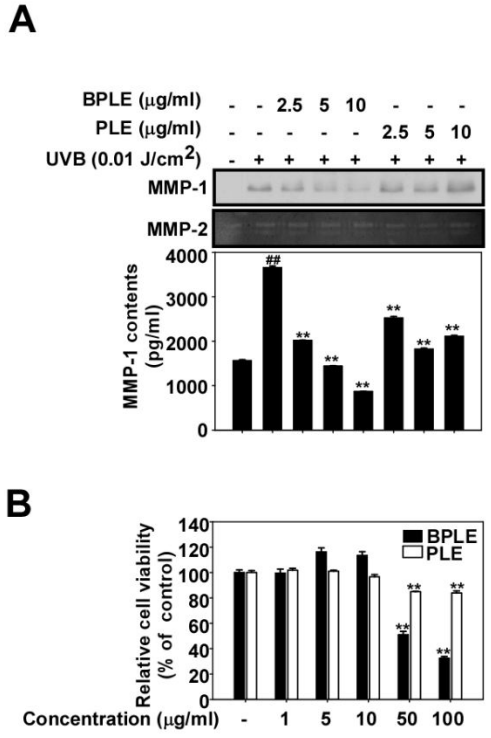

A
BPLE (μg/ml) - - 2.5 5 10 - - -
PLE (μg/ml) - - - - - 2.5 5 10
UVB (0.01 J/cm2) - + + + + + + +
MMP-1
MMP-2
MMP-1 contents (pg/ml)
4000
3000
2000
1000
0
##
**
B
Relative cell viability (% of control)
140
120
100
80
60
40
20
0
BPLE
PLE
Concentration (μg/ml) - 1 5 10 50 100

Figure 1. BPLE has stronger inhibitory effects against UVB-induced MMP-1 expression than that of PLE in human keratinocyte

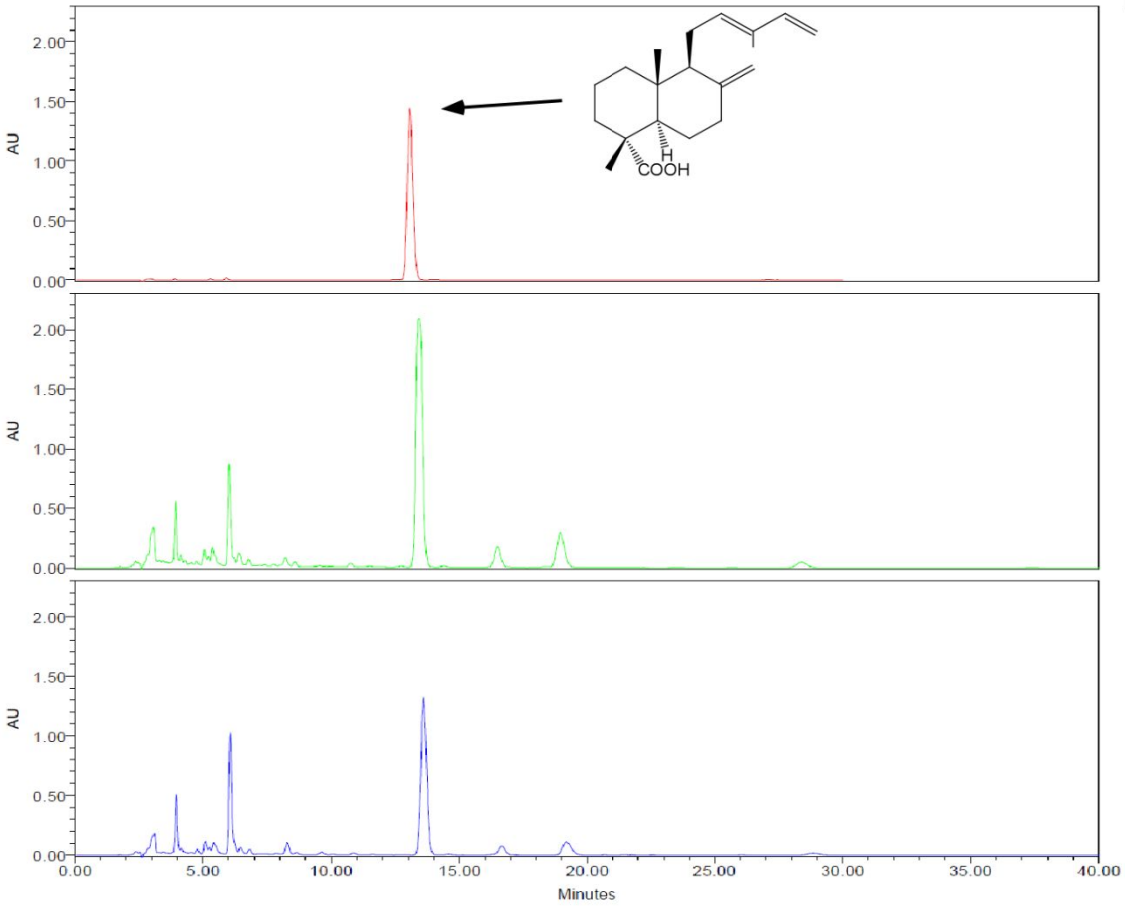
A. Brown pine leaf extract (BPLE) has stronger inhibitory effects against ultraviolet B (UVB)-induced matrix metalloproteinase-1 (MMP-1) expression than that of pine leaf extract (PLE) in human keratinocyte. Cells were treated with BPLE and PLE at the indicated concentration for 1h before being exposed to 0.01 J/cm^2 of UVB and media was harvested 48 h later. Protein expression was analyzed by Western blotting (MMP-1), Zymography (MMP-2), and ELISA (MMP-1 contents). B. Cell viability in human keratinocyte. Cell viability was measured using MTT assay as described in Materials and Methods. For A and B, data are presented as the mean ±S.D. of MMP-1 protein content and cell viability as determined for three independent experiments. The asterisk indicated a significant difference (#, $p < 0.05$, ##, $p < 0.01$) between the UVB-treated group and the untreated control group, (*, $p < 0.05$, **, $p < 0.01$) between the group treated with BPLE or PLE and the untreated control group.

3.2. TCA and DAA increased in the color change process of pine leaf from green to brown

Because BPLE has stronger inhibitory effect against UVB-induced MMP-1 protein expression than that of PLE, it is hypothesized that an active component is produced during the pine leaf color changing process. According to chemical profiling by High performance liquid chromatography (HPLC), TCA and DAA are detected as 90.5 mg/g and 14.26 mg/g in PLE respectively. However, the quantity of TCA and DAA are highly detected as 180.62 mg/g, 60.3 mg/g in BPLE (Fig. 2A and B).

Figure 2

A


AU
2.00
1.50
1.00
0.50
0.00
COOH
H
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
Minutes

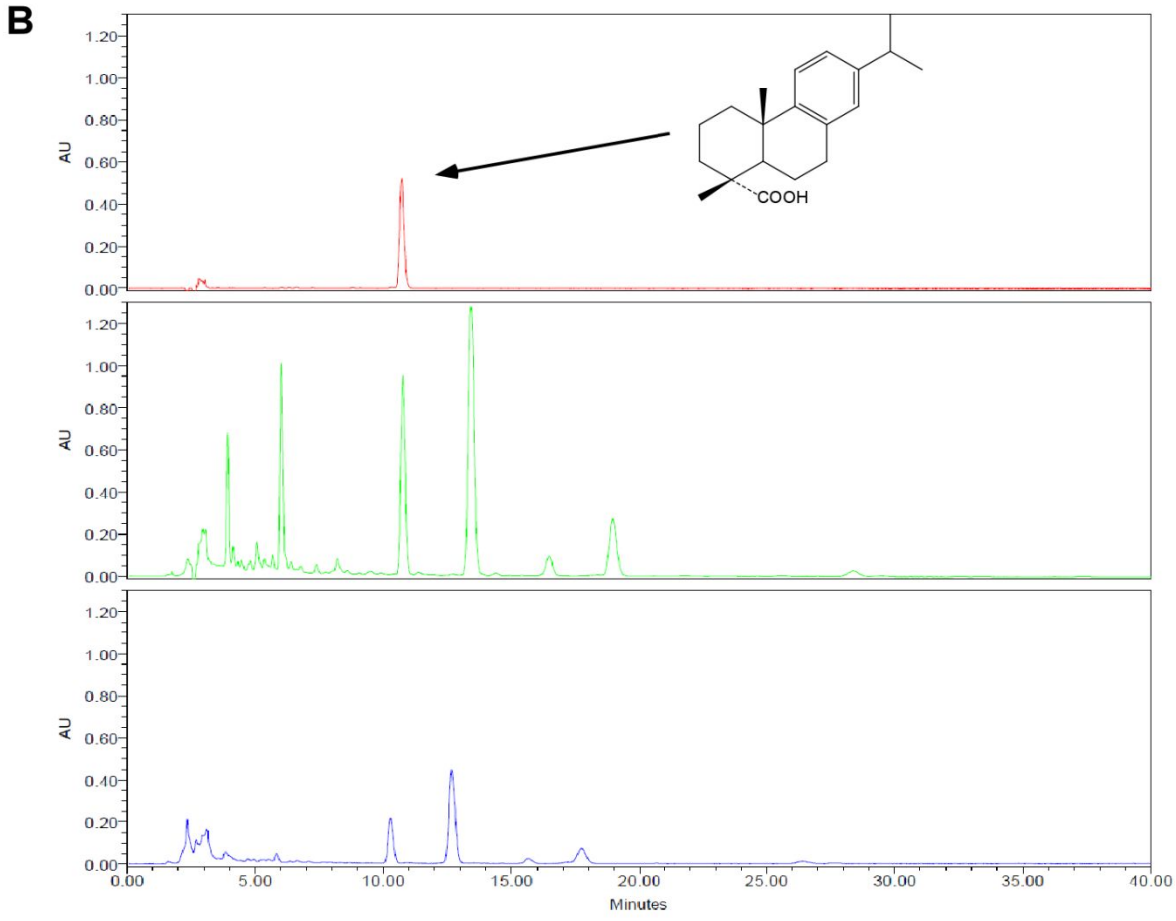

B
AU
1.20
1.00
0.80
0.60
0.40
0.20
0.00
COOH
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
Minutes

Figure 2. TCA and DAA increased in the color change process of pine leaf from green to brown

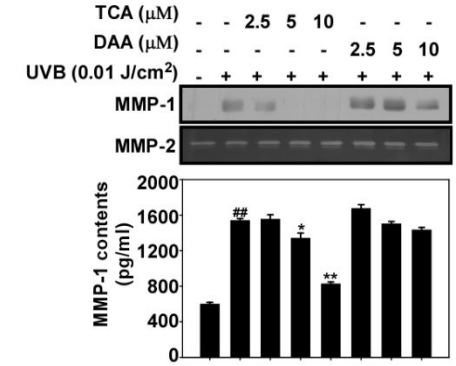
A and B. Isolation and characterization of *trans*-communic acid (TCA) (A) and dehydroabietic acid (DAA) (B) from BPLE and PLE. BPLE and PLE (0.02 g) was prepared in ethanol (80 %). Isolation was conducted as described in Materials and Methods. BPLE appears as green and PLE is blue.

3.3. TCA suppressed UVB-induced MMP-1 expression in human keratinocyte but DAA did not

To verify the inhibitory effect of TCA and DAA against UVB-induced MMP-1 expression, the effect of TCA and DAA against UVB-induced MMP-1 protein expression in human keratinocyte were examined within the range of concentration which does not show cytotoxicity (Fig. 3B). Results showed that TCA inhibited UVB-induced MMP-1 protein expression in a dose dependent manner (Fig. 3A). Therefore, TCA is suggested to be a major active component of BPLE.

Figure 3

A


TCA (μM) - - 2.5 5 10 - - -
DAA (μM) - - - - - 2.5 5 10
UVB (0.01 J/cm2) - + + + + + + +
MMP-1
MMP-2
MMP-1 contents (pg/ml)
2000
1600
1200
800
400
0
##
*
**

B

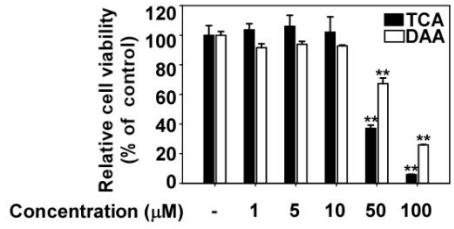

Relative cell viability (% of control)
120
100
80
60
40
20
0
TCA
DAA
**
Concentration (μM) - 1 5 10 50 100

Figure 3. TCA suppressed UVB-induced MMP-1 expression in human keratinocyte but DAA did not

A. TCA and DAA inhibits UVB-induced MMP-1 protein expression than in human keratinocyte. Cells were treated with TCA and DAA at the indicated concentration for 1h before being exposed to 0.01 J/cm^2 of UVB and media was harvested 48 h later. Protein expression was analyzed by Western blot assay (MMP-1), Zymography (MMP-2), and ELISA (MMP-1 contents) B. Cell viability in human keratinocyte. Cell viability was measured using MTT assay as described in Materials and Methods. For A and B, data are presented as the mean ±S.D. of MMP-1 protein content and cell viability as determined for three independent experiments. The asterisk indicated a significant difference (#, $p < 0.05$, ##, $p < 0.01$) between the UVB-treated group and the untreated control group, (*, $p < 0.05$, **, $p < 0.01$) between the group treated with TCA or DAA and the untreated control group.

3.4. BPLE and TCA inhibited UVB-induced MMP-1 mRNA expression and AP-1 transactivation in human keratinocyte

The inhibitory effect of BPLE and TCA against MMP-1 mRNA expression and AP-1 transactivation were evaluated. BPLE and TCA inhibited UVB-induced MMP-1 mRNA expression dose dependently (Fig. 4A and B). It is reported that AP-1 is a major transcription factor of UVB-induced MMP-1 expression [3, 25-27]. The inhibitory effect of BPLE and TCA against UVB-induced AP-1 transactivation was examined by using HaCaT cells stably transfected with AP-1 luciferase plasmid. The results showed that BPLE and TCA attenuated UVB-induced AP-1 transactivation (Fig. 4C and D).

Figure 4

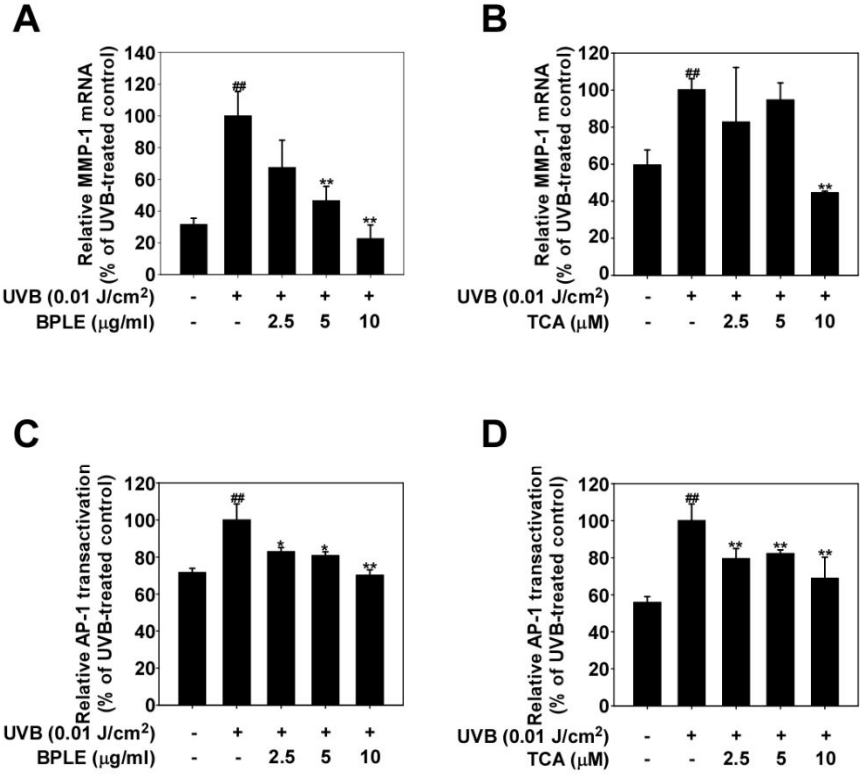

A
Relative MMP-1 mRNA (% of UVB-treated control)
140
120
100
80
60
40
20
0
##
**
**
UVB (0.01 J/cm2) - + + + +
BPLE (μg/ml) - - 2.5 5 10
B
Relative MMP-1 mRNA (% of UVB-treated control)
120
100
80
60
40
20
0
##
**
UVB (0.01 J/cm2) - + + + +
TCA (μM) - - 2.5 5 10
C
Relative AP-1 transactivation (% of UVB-treated control)
120
100
80
60
40
20
0
##
*
*
*
UVB (0.01 J/cm2) - + + + +
BPLE (μg/ml) - - 2.5 5 10
D
Relative AP-1 transactivation (% of UVB-treated control)
120
100
80
60
40
20
0
##
**
**
**
UVB (0.01 J/cm2) - + + + +
TCA (μM) - - 2.5 5 10

Figure 4. BPLE and TCA inhibited UVB-induced MMP-1 mRNA expression and AP-1 transactivation in human keratinocyte

A and B. BPLE and TCA inhibits UVB-induced MMP-1 mRNA expression in human keratinocyte. Cells were treated with BPLE and TCA at the indicated concentration for 1 h before being exposed to 0.01 J/cm^2 of UVB and mRNA was collected 12 h later. MMP-1 mRNA expression was analyzed as Quantitative RT-PCR described in the Materials and Methods. C and D. BPLE and TCA attenuated UVB-induced AP-1 transactivation in human keratinocyte. Cells were treated with BPLE and TCA at the indicated concentration for 1 h before being exposed to 0.01 J/cm^2 of UVB and cells were lysed 12 h later. AP-1 transactivation was measured using Luciferase assay described in the Materials and Methods. From A to D, data are presented as the mean ±S.D. of MMP-1 mRNA expression and AP-1 transactivation as determined for three independent experiments. The asterisk indicated a significant difference (#, $p < 0.05$, ##, $p < 0.01$) between the UVB-treated group and the untreated control group, (*, $p < 0.05$, **, $p < 0.01$) between the group treated with BPLE or TCA and the untreated control group.

3.5. BPLE and TCA did not affect UVB-induced cellular signal transduction which regulates AP-1 in human keratinocyte

MAPK cellular signal pathways play crucial role in UVB-induced AP-1 transactivation [15, 28, 29]. The inhibitory effect of BPLE and TCA in UVB-induced MAPK signal transduction was examined. Results showed that both BPLE and TCA have no significant effect on MAPK signal transduction. (Fig. 5A and B).

Figure 5

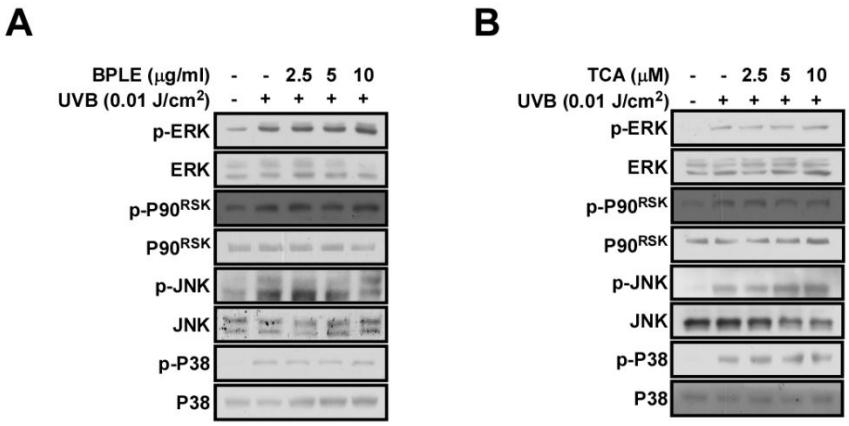

A
BPLE (μg/ml) - - 2.5 5 10
UVB (0.01 J/cm2) - + + + +
p-ERK
ERK
p-P90RSK
P90RSK
p-JNK
JNK
p-P38
P38
B
TCA (μM) - - 2.5 5 10
UVB (0.01 J/cm2) - + + + +
p-ERK
ERK
p-P90RSK
P90RSK
p-JNK
JNK
p-P38
P38

Figure 5. BPLE and TCA did not affect UVB-induced cellular signal transduction which regulates AP-1 in human keratinocyte

A and B. Both of BPLE and TCA have no significant effect on MAPK signal transduction. Cells were treated with BPLE and TCA at the indicated concentration for 1 h before being exposed to 0.01 J/cm^2 of UVB and lysed 30 min. Phosphorylated and total protein levels were conducted by Western blot assay using specific antibodies described in the Materials and Methods.

3.6. BPLE and TCA reduced collagen degradation and attenuated MMP-1 expression in human skin equivalent model

To further investigate anti-photoaging effect of BPLE and TCA, human skin equivalent model was used. Their effect were examined by masson's trichrome staining and immunohistochemical staining. The results found preventive effect of BPLE (5, 10 μg/ml) and TCA (5, 10 μM) against UVB-induced collagen degradation and MMP-1 overexpression in human skin equivalent model (Fig. 6A and B).

Figure 6

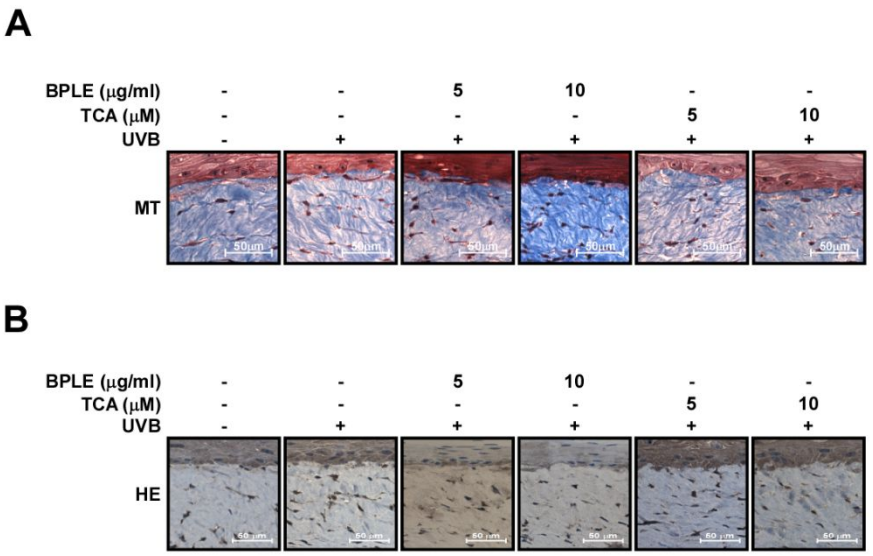

A
BPLE (μg/ml) - - 5 10 - -
TCA (μM) - - - - 5 10
UVB - + + + + +
MT
50 μm
B
BPLE (μg/ml) - - 5 10 - -
TCA (μM) - - - - 5 10
UVB - + + + + +
HE
50 μm

Figure 6. BPLE and TCA reduced collagen degradation and attenuated MMP-1 expression in human skin equivalent model

A. BPLE and TCA inhibits UVB-induced MMP-1 protein expression levels and collagen degradation. The serial sections, from the human skin equivalent, were mounted into silane-coated slides and subjected to immunohistochemical staining using anti-MMP-1 antibody (A) or masson trichrome staining (B) as described in the Materials and Methods. MMP-1 appears brown.

IV. Disccusion

Human skin equivalent model is kinds of three dimensional cell culture system which is suggested to solve the limit of two dimensional cell culture system. In current studies, these three dimensional cell culture system often are used to research diseases such as, netherton syndrome [30], ichthyosis [31] and skin inflammation [32]. It has advantage in clinical relevance and free to animal welfare. I evaluate effect of BPLE and TCA on UVB- induced MMP-1 expression in human skin equivalent model. The results showed that UVB-induced collagen degradation and MMP-1 expression were decreased in BPLE and TCA treated human skin equivalent.

trans-communic acid is a group of diterpenic acid with a labdane skeleton containing three double bonds and a carboxyl group. There are five communic acid that differ in the location of the double bonds and the orientation of the carboxyl group. above all, *trans*-communic acid most abundant in nature. It is widely distributed in Cupresaceae species, especially in the genus Juniperus [33]. Previous study report that it exhibit anti-mycobacterial [34], anti-tumoral [35, 36], anti-inflammatory,

and antioxidant [37]. In this study, I identify TCA is a dominant chemical in BPLE. Both of BPLE and TCA have anti-photoaging effect and show similar mechanism. Therefore, TCA is a major active component in BPLE.

AP-1 is a major transcription factor in UVB-induced MMP-1 expression [3, 25-27]. It binds to the TPA-response element (TRE) and activates transcription of target gens [18, 38]. AP-1 complex consist of a lot of subunit, and they are have different roles. For example, c-Jun is and efficient activator of MMP-1 transcription but not JunB which act antagonistically. And, MMP-1 promoter binding affinity is different as consist of AP-1 subunit. AP-1 activity is affect by cofactor like Jun activation domain-binding protein (JAB1), CREB-binding protein (CBP) [39, 40]. BPLE and TCA attenuate UVB-induced AP-1 transactivation. To further investigate direct target, BPLE and TCA have effect on which AP-1 subunit or cofactor should be identified.

In summary, our results demonstrate that BPLE has more inhibitory effects in UVB-induced MMP-1 expression than that of PLE in human keratinocyte. It seems that this effect is based on TCA. BPLE and

TCA attenuated UVB-induced MMP-1 mRNA expression and AP-1 transactivation. Futhermore, BPLE and TCA decrease UVB-induced collagen degradation and MMP-1 expression in human skin equivalent model.

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VI. 국문초록

소나무는 국송으로서 우리 나라 전국 각지에 분포한다. 솔잎은 2 년을 주기로 생장하여 갈색화되어 떨어져 이용되지 않고 버려진다. 본 연구에서는 갈색솔잎은 유용한 기능성 소재로 활용하기 위해 자외선으로 유도되는 광노화 예방 소재로서 효능을 규명하였다. 갈색솔잎추출물은 인간각화세포에서 UVB 에 의해 유도된 matrix metalloproteinase-1 (MMP-1)의 발현을 솔잎추출물보다 농도의존적으로 더 억제하였다. 이러한 갈색솔잎추출물의 효능의 증가를 규명하기 위해 성분 분석을 수행한 결과 갈색솔잎추출물에서 녹색솔잎추출물 보다 *trans*-communic acid (TCA)와 dehydroaibetic acid (DAA)가 각각 2 배와 4 배 많이 검출되었다. UVB 에 의해

유도되는 MMP-1 발현 억제효능을 확인한 결과 TCA 만이 효능이 있었고 두 물질의 작용기전을 동시에 분석하였다. 갈색솔잎추출물과 TCA 모두 자외선으로 유도되는 MMP-1 mRNA 와 AP-1 전사활성을 유의적으로 억제하였다. 또한, AP-1 전사활성에 관여하는 상위 분자기전인 MAPK 인산화단백질에 영향을 미치지 않음을 확인하였다. 또한, 생리학적으로 유사한 효능 검증을 위해 생체피부모방모델에서 콜라겐 분해정도와 MMP-1 발현을 확인한 결과 갈색솔잎추출물과 TCA 모두 유의적으로 억제하는 것을 확인하였다.

본 연구 결과를 통하여 갈색솔잎추출물이 UVB 로 유도되는 피부노화를 억제하는 효능을 가지고 있음을 규명하였고, TCA 와 작용기전 분석을 통하여 갈색솔잎추출물의

유효성분이 TCA 임을 규명하였다. 따라서, 갈색솔잎추출물은 기존의 버려지던 자원에서 벗어나 광노화 억제 효능을 가지는 신규 소재로써 활용 할 수 있을 것이다.