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잘피(*Zostera marina* L.)의 신규 항노화 화장품 소재 응용

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New Cosmetic Agents for Anti-aging from *Zostera marina* L.

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요 약: 해양 천연물 유래의 신규 광노화 방지소재의 개발을 위해 항산화 활성과 matrix metalloproteinase-1 (MMP-1) 발현 억제활성을 갖는 잘피(*Zostera marina* L.)를 선별하였다. 에탄올 추출물로부터 3개의 화합물(compound 1과 2, 3)을 분리 하였으며 각각 apigenin-7-*O*-β-D-glucoside (1)과 chrysoeriol (2), luteolin (3)으로 동정하였다. 이 화합물들은 1,1-diphenyl-2-picryl-hydrazyl radical에 대하여 각각 0.18 mM과 0.68 mM, 0.01 mM의 SC_{50} 값을 나타내었으며, xanthine과 xanthine oxidase의 반응으로 생성되는 superoxide radical에 대하여 각각 0.04 mM과 0.03 mM, 0.01 mM의 SC_{50} 값을 나타내었다. 특히 compound 3은 MMP-1에 대해 35.0 μM의 농도에서 44% 이상의 발현 억제활성을 나타내었으며 MMP-1 발현을 유도하는 신호전달물질로 알려져 있는 interleukin 6의 생성도 억제하였다. 또한 잘피 추출물을 함유한 제품이 주름 개선효과를 측정하였다. 추출물을 3.0% 함유한 크림을 8주간 적용하여 미세주름과 피부거칠음의 현저한 개선효과를 확인하였다. 결론적으로, 잘피 추출물에서 분리된 화합물들은 우수한 항산화 활성과 MMP-1 발현 억제활성을 가지며, 이 추출물을 함유한 제품은 피부주름의 감소효과를 나타내었다. 따라서, 잘피 추출물은 화장품의 새로운 항노화 소재로서 적용될 수 있을 것이다.

Abstract: In order to develop new anti-photoaging agents from marine natural products, *Zostera marina* L. was selected for its antioxidative activity and inhibition of matrix metalloproteinase-1 (MMP-1) expression. Three compounds (compounds 1, 2, and 3) were isolated from the extract, and they were identified as apigenin-7-*O*-β-D-glucoside (1), chrysoeriol (2), and luteolin (3). These compounds have SC_{50} values of 0.18 mM, 0.68 mM, and 0.01 mM against 1,1-diphenyl-2-picrylhydrazyl radical and 0.04 mM, 0.03 mM, and 0.01 mM against the superoxide radical in the xanthine/xanthine oxidase system, respectively. Compound 3 suppressed the expression of MMP-1 by up to 44% at 35.0 μM and inhibited the production of interleukin 6, which is known as a cytokine that induces MMP-1 expression. In addition, the wrinkle improvement effect of the formulation with *Z. marina* extract was measured. As a result, remarkable reduction was found in the fine wrinkle and skin roughness after application of the cream with 3.0% this extract for 8 weeks. In conclusion, the isolated compounds from *Z. marina* extract were good antioxidant and suppressor of MMP-1 expression and the formulation with the extract diminished the skin wrinkle. Therefore, the extract can be used as a new anti-aging agent for application in cosmetic.

Keywords: *ani-aging, antioxidant, matrix metalloproteinase-1, Zostera marina L., luteolin*

1. Introduction

The aging of human skin has two elements: changes that occur in all individuals with the passage of time and changes that occur in varying degrees to different individuals as a result of repeated environmental exposure. The primary environmental factor is UV irradiation from the sun[1], which produces free radicals and related reactive oxygen species (ROS). These injure the DNA and extracellular matrix (ECM) and cause peroxidation of membrane lipids in skin cells[2,3]. In addition, UV irradiation has been shown to stimulate the overexpression of genes of matrix metalloproteinases (MMPs) by cytokines, such as interleukins (IL), through DNA damage or the generation of ROS[4]. Researches

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have shown that MMP-1 derived from fibroblasts is induced dose-dependently *in vitro* and *in vivo* by UV irradiation, and this induction was at least partly mediated by IL-1α/β and IL-6[4].

MMPs constitute a large family of proteases that have been identified and classified in more than 20 species. These enzymes can degrade most components of the ECM such as collagens, laminins, fibronectins, elastins[5]. Histological studies have revealed that the major alterations in photoaged skin are localized in the connective tissue[6]. Collagen constitutes about 90% (in dry weight) of skin connective tissue, and MMP-1 is a specific enzyme to collagens. Thus, UV irradiation may cause damage by disorganizing of collagen fibrils by MMP reaction, especially MMP-1[7]. Since collagen fibrils with elastin are responsible for the strength and resiliency of skin, their disarrangement causes wrinkles and skin aging.

In order to develop anti-photoaging agents, we focused on the ability to scavenge free radicals and ROS and suppress MMP expression and related cytokine production. In the course of screening for anti-photoaging agents in marine plants, we found the extract of *Zostera marina* L. leaves to show significant activity. *Z. marina* is a seagrass that lives in the nearshores of East Asia, Europe, and North America[8].

Based on these premises, we isolated and structure determined of active compounds, and examined biological effects against DPPH, superoxide radicals, and MMPs. In addition, *Z. marina* extract might be expected to benefit photo-damaged skin, including the fine wrinkles and roughness, we applied as an anti-aging agents.

2. Materials and Methods

2.1. Extraction and Isolation

Z. marina leaves were collected at the western shores of Korea in February 2002. Dried *Z. marina* leaves (250.0 g) were refluxed with 70% aqueous ethanol and the extract was evaporated. The extract (75.0 g) was suspended in water and the suspension was partitioned with hexane (24.0 g), CH_2Cl_2 (11.7 g), EtOAc (2.9 g), and butanol (9.4 g), consecutively. The EtOAc extract (2.8 g) was chromatographed on a Sephadex LH-20 column (3.1×45 cm, 40→100% MeOH) to afford 18 subfractions (Ⅰ~ⅩⅧ). Compounds **1** (40 mg) and **3** (90 mg) were obtained by recrystallyzation in MeOH from the subfractions Ⅶ and ⅩⅦ, respectively. Purification of subfraction ⅩⅢ on a TLC plate (kieselgel 60 F_{254}, CH_2Cl_2 : MeOH = 10 : 1.5) yielded compound **2** (35 mg).

2.2. Compound 1 (apigenin-7-O-β-D-glucoside)

Amorphous pale yellowish powder; $FeCl_3$ Positive; $C_{21}H_{20}O_{10}$ (M. w. 432.39); EIMS *m/z*: 432 $[M]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): 7.97 (2H, d, J = 9.0 Hz, H-2′, 6′), 6.95 (2 H, d, J = 9.0 Hz, H-3′, 5′), 6.87 (1 H, s, H-3), 6.84 (1 H, d, J = 2.0 Hz, H-8), 6.45 (1 H, d, J = 2.0 Hz, H-6), 5.08 (1 H, d, J = 7.5 Hz, H-1″), 3.73 (1 H, m, H-6″), 3.51 (1 H, m, H-6″), 3.45~3.20(3 H, m, H-2″, 3″, 4″); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: Table 1.

2.3. Compound 2 (chrysoeriol, luteolin 3′-methyl ether)

Amorphous pale yellowish powder; $FeCl_3$ Positive; $C_{16}H_{12}O_6$ (M. w. 300.27); EIMS *m/z*: 300 $[M]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): 7.58 (1 H, d, J = 9.0 Hz, H-6′), 7.57 (1 H, s, H-2′), 6.95 (1 H, d, J = 9.0 Hz, H-5′), 6.90 (1 H, s, H-3), 6.52 (1 H, d, J = 1.5 Hz, H-6), 6.20 (1 H, d, J = 1.5 Hz, H-8), 3.90 (3 H, s, OCH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: Table 1.

2.4. Compound 3 (luteolin)

Amorphous pale yellowish powder; $FeCl_3$ Positive; $C_{15}H_{10}O_6$ (M. w. 286.24); EIMS *m/z*: 286 $[M]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): 7.43 (1 H, dd, J = 2.0, 8.0 Hz, H-6′), 7.40 (1 H, d, J = 2.0 Hz, H-2′), 6.90 (1 H, d, J = 8.0 Hz, H-5′), 6.67 (1 H, s, H-3), 6.45 (1 H, d, J = 2.0 Hz, H-6), 6.20 (1 H, d, J = 2.0 Hz, H-8); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: Table 1.

2.5. Measurement of Antioxidative Activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect was evaluated according to the method of Blois, *et al.* with minor modifications[9]. And the scavenging activity on the ROS was measured by monitoring the reduction of nitroblue tetrazolium (NBT)[10].

2.6. UV Irradiation and Detection of MMP-1 and Cytokines by Enzyme Linked Immunosorbent Assay (ELISA)

Human skin fibroblasts (Hs68) and keratinocyte cell lines (HaCaT) were purchased from the American Type Culture Collection. The cells were cultured in Dulbecco's

Table 1. ^{13}C-NMR Data of Compounds **1**, **2**, and **3** Isolated from *Zostera marina*[a]

No.	^{13}C (δ) in DMSO- d_6		
	1	2	3
2	164.74	164.89	164.88
3	103.59	104.46	103.64
4	182.48	182.56	182.42
5	161.84	162.19	162.24
6	100.00	99.57	99.59
7	163.44	164.42	164.66
8	95.32	94.80	94.60
9	157.42	158.09	158.05
10	105.82	103.97	104.50
1′	121.51	122.27	122.27
2′	129.09	111.00	114.12
3′	116.47	151.47	146.49
4′	161.59	148.78	150.45
5′	116.47	116.51	116.77
6′	129.09	121.12	119.75
1″	100.38		
2″	73.57		
3″	76.91		
4″	70.03		
5″	77.65		
6″	61.08		
Methoxy-C		56.72	

[a] TMS was used as internal standard; the data of compounds **1**, **2**, and **3** were obtained at 75 MHz. DMSO-d_6 was used as the solvent.

Modified Eagle's Medium with 10% fetal bovine serum and incubated in a humidified 5% CO_2 incubator at 37℃. The expression of MMP-1 and cytokines (IL-1α and IL-6) induced by UVA and UVB irradiation was estimated by ELISA, modifying the reported methods [11-13].

2.7. Measurement of Wrinkle Improvement Effect of *Z. marina* Extract

The wrinkle improvement effect of the formulation with 3.0% *Z. marina* extract was measured using the Skin Visiometer SV600. Twenty volunteers were asked to apply the formulation containing *Z. marina* extract to one side of face, and the placebo to the other side once and above for 8 weeks. A silicone replica of the crow's feet area was taken at the 0 and 8 weeks, respectively. Standard wrinkle and roughness were calculated by the previous reported methods[14].

2.8. Statistical Analysis

The results of ELISA assay of MMP-1, IL-1α, and IL-6 were expressed as means±S.D. from three separate experiments. The Student's t-test was used to evaluate the differences of the means between the control and the samples, accepting $p < 0.05$ as significant.

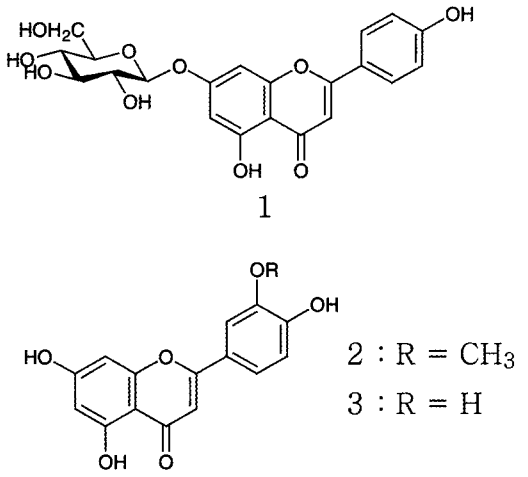

Figure 1. Structures of compounds **1**, **2**, and **3** isolated from *Z. marina*.

3. Results and Discussion

The activity-guided purification of EtOAc soluble fraction was afforded three compounds - compounds **1**, **2**, and **3**. These compounds were obtained as an amorphous pale yellowish powder, positive to $FeCl_3$, and showed $[M]^+$ at *m/z* 432, 300, and 286 in EIMS spectrum, respectively. From spectral data of ^1H- and ^{13}C-NMR, the structures were identified as apigenin-7-*O*-β-$_D$-glucoside, chrysoeriol, and luteolin, respectively. Finally, these were confirmed by comparing its NMR data with those in the reported references[15-18]. The structures of compounds **1**, **2**, and **3** are presented in Figure 1.

The three compounds isolated from *Z. marina* showed strong antioxidative activity. The SC_{50} (the concentration of the sample required for 50% of the free radicals to be scavenged) values of compounds **1**, **2**, and **3** against the DPPH radical were 0.18 mM, 0.68 mM, and 0.01 mM, respectively. As summarized in Table 2, the SC_{50} of compound **3** was stronger than that of vitamin C (0.06 mM), vitamin E (0.03 mM), and 3-*t*-butyl-4-hydroxyanisole (BHA; 0.08 mM). The SC_{50} values of the three compounds against superoxide radical in the

Table 2. Radical Scavenging Activity of Compounds **1**, **2**, and **3** Isolated from *Z. marina* (against DPPH and Superoxide Radical)

Compounds	SC_{50} values (mM)[a]	
	DPPH[b]	Superoxide radical[c]
1	0.18	0.04
2	0.68	0.03
3	0.01	0.01
BHA[d]	0.08	0.18
Vitamin C	0.06	ND[e]
Vitamin E	0.03	ND

[a] SC_{50} value, sample concentration to scavenge reactive oxygen species by 50%. [b]DPPH, 1,1-diphenyl-2-picrylhydrazyl radical. [c] Superoxide radical was produced from xanthine/xanthine oxidase oxidation reaction. [d]BHA, 3-*t*-butyl-4-hydroxyanisole. [e]ND, Not determined.

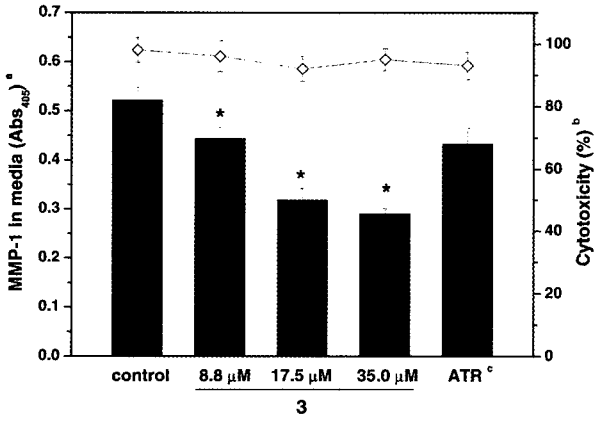

Figure 2. The suppression activity of compound **3**, isolated from *Z. marina*, on the expression of matrix metalloproteinase-1. [a]MMP-1, matrix metalloproteinase-1 (■). The MMP-1 contents of culture media were determined by ELISA. [b]Cytotoxicity was measured by MTT assay (-◇-). The viability of cells was expressed as a percentage. [c]ATR, all-*trans*-retinol. The concentration of all-*trans*-retinol was 4.0 μM. Means±S.D. and t-test significance levels were calculated on the relative values and are presented when the number of wells was 3. $*p < 0.05$ compared with control.

xanthine/xanthine oxidase system were 0.04 mM, 0.03 mM, and 0.01 mM, respectively. Their activities were stronger than that of BHA, which was used as a positive control (Table 2).

Of the three compounds, compound **3** exhibited the most potent antioxidative activity, and the effects of compound **3** on MMP-1 suppression was measured and

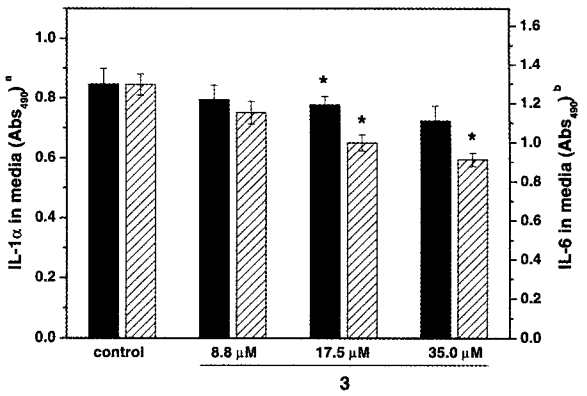

Figure 3. The suppression activity of compound **3**, isolated from *Z. marina*, on interleukins secretion. [a]IL-1 α, interleukine 1 α (■). [b]IL-6, interleukine 6 (▨). The cytokine content of the culture media was determined by ELISA. Means±S.D. and t-test significance levels were calculated on the relative values and are presented when the number of wells was 3. $*p < 0.05$ compared with control.

compared with that of retinoid, a positive control. The inhibitory activity of compound **3** was 44% at 35.0 μM, while that of the positive control, all-*trans*-retinol was 17% at the same concentration in human skin fibroblasts (Figure 2).

In addition, since interleukins are known to stimulate the expression of MMP-1, we tested the effect of compound **3** on them. Compound **3** inhibited the suppression of IL-6 expression by 30% at 35.0 μM, but IL-1 α was weak (Figure 3). Therefore, the inhibitory activity of compound **3** on interleukins production may have an effect on suppressing MMP-1.

From these results, the *Z. marina* extract was expected the reduction effect of the fine wrinkle that have antioxidants and inhibitor of MMP-1, we estimated the efficacy of the formulation with the extract using Skin Visiometer SV600[14]. As summarized in Figure 4, after 8 weeks of application of placebo, values of parameter (R1~R5) were not showed a significant difference as compared with initial values. However, the case of application of formulation with 3.0% *Z. marina* extract, the values of R1, R2, and R5 was showed remarkable reduction. These data suggest that *Z. marina* extract effectively decreased facial wrinkle compared with placebo after 8 weeks of use.

In conclusion, three compounds isolated from *Z.*

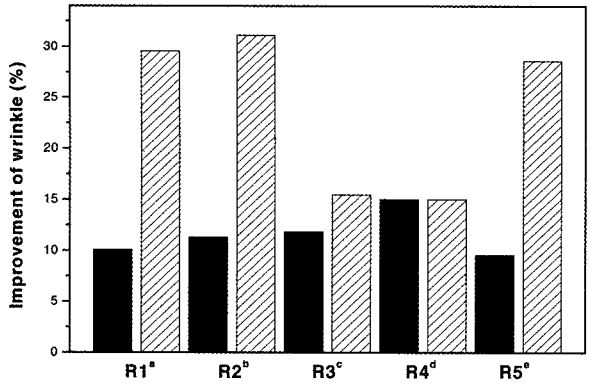

Figure 4. The improvement effect of the formulation with 3.0% *Z. marina* extract on fine wrinkle. Placebo, the same formulation without the extract (■). The formulation with 3.0% *Z. marina* extract (▨). [a]R1, skin roughness. [b]R2, maximum roughness. [c]R3, average roughness. [d]R4, smoothness depth. [e]R5, arithmetic average roughness. The wrinkle improvement effect of the formulation with 3.0% *Z. marina* extract was measured using the skin visiometer SV600. Twenty volunteers were asked to apply the formulation containing the extract to one side of face, and the placebo to the other side once and above for 8 weeks. A silicone replica of the crow's feet area was taken at the 0 and 8 weeks, respectively. Standard wrinkle and roughness were calculated from values of parameters, R1~R5.

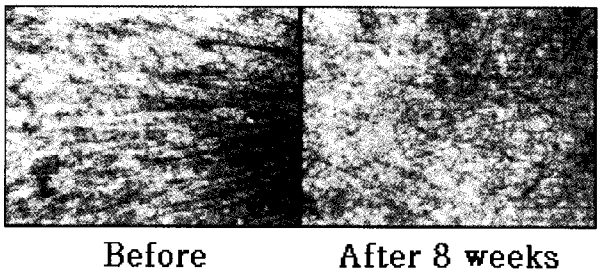

Figure 5. The pictures of the wrinkle improvement of the formulation with 3.0% *Z. marina* extract. The pictures were obtained by visioscan (C+K).

marina extract had strong antioxidative activity. In particular, compound **3** suppressed the expression of MMP-1 because of its antioxidative effect and by inhibiting IL-1α and IL-6 production. In addition, the extract containing these active compounds was improved the fine wrinkle and skin roughness. Therefore, these compounds and *Z. marina* extract are expected to be useful in protecting skin aging from UV irradiation.

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