The Inhibitory Effects of Eckol and Dieckol from *Ecklonia stolonifera* on the Expression of Matrix Metalloproteinase-1 in Human Dermal Fibroblasts

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In order to develop new anti-photoaging agents, we examined the inhibitory effects of 29 seaweed extracts on transcriptional activities of NF-κB and AP-1, and MMP-1 expression. The extracts from 3 species of Alariaceae, *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia stolonifera*, have showed strong inhibition of both NF-κB and AP-1 reporter activity, which were well correlated with their abilities to inhibit MMP-1 expression. In addition, MMP-1 expression was dramatically attenuated by treatment with eckol or dieckol which were purely isolated from *E. stolonifera*, indicating that these compounds are active principles to inhibit MMP-1 expression in human dermal fibroblasts. Taken together, our data demonstrate the inhibitory effect of eckol and dieckol from *Ecklonia* species on MMP-1 expression in human dermal fibroblasts and provide a possibility to develop as an agent for the prevention and treatment of skin aging.

Key words *Ecklonia stolonifera*; eckol; dieckol; matrix metalloproteinase (MMP)-1; NF-κB; AP-1

The skin aging process can be considered to be due to intrinsic aging and photoaging.[1—3)] The intrinsic aging is due to chronologic damage caused by slow and irreversible tissue degeneration whereas the photoaging is primarily the results of UV exposure.[2,3)] Clinically, chronologically aged skin is smooth, pale, and finely wrinkled. In contrast, photoaged skin is coarsely wrinkled and associated with dyspigmentation and telangiectasia.[1,4)]

Alterations of collagen, the major structural component of skin, in dermis layer have been suggested to be causes of the clinical changes observed in naturally aged and photoaged skin.[5,6)] This collagen deficiency may arise from its reduced synthesis as well as increased degradation with a concomitant elevation of matrix metalloproteinase (MMP) expression. Ultraviolet irradiation induces the synthesis of MMP in human skin *in vivo*, and MMP-mediated collagen destruction accounts, in large part, for the connective tissue damage that occurs in photoaging.[7)] Therefore, it is believed that the restoration of the collagen deficiency in aged human skin by the induction of new collagen synthesis and/or by the reduction of MMP will present a possible strategy for preventing and treating the clinical manifestations of skin aging, namely, wrinkles and skin laxity. MMPs can be divided into four categories based on the substrate preference: collagenases, gelatinases, stromelysins, and membrane-type MMPs.[8)] Among human MMPs reported previously, MMP-1, which is an interstitial collagenase, is mainly responsible for the degradation of dermal collagen in human skin aging process.[9,10)]

Marine natural products provide a rich source of chemical diversity that can be used to design and develop new, potentially useful therapeutic agents. In this study, therefore, we tried to screen active compounds from 29 marine natural products which would be able to inhibit MMP-1 expression in human dermal fibroblasts and isolated eckol and dieckol from *Ecklonia stolonifera* which are major and active compounds to inhibit MMP-1 expression.

MATERIALS AND METHODS

Primary Human Dermal Fibroblast Cell Culture and Cytotoxicity Test Primary human foreskin fibroblasts were established by outgrowth from biopsies of healthy donor of 10 years old, which was performed under the consent of volunteer, and cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37 °C. Cultured cells at passages 4—10 were used for the experiments. For treatment, primary human dermal fibroblasts were maintained on culture media without FBS for 48 h. Cytotoxicity of cells was determined by the MTS assay (Promega, Madison, WI, U.S.A.) according to the manufacturer's instruction.

Preparation of Seaweed Extracts Twenty nine different species of seaweed were harvested in the seashore in East Sea, Korea, and identified. The seaweeds were homogenized and crude components were extracted three times with methanol. The filtered supernatants were solubilized with appropriate amount of water and re-extracted with ethyl acetate. Ethyl acetate soluble fractions were evaporated under a vacuum at 45 °C, dissolved with DMSO and stored at −70 °C until using in other experiments. The amount of each extracts was presented in Table 1.

Isolation of Eckol and Dieckol from *E. stolonifera* Two kilograms of *E. stolonifera* was homogenized and the crude components were extracted three times with methanol and evaporated under a vacuum at 45 °C. The extract was solubilized with 2 l of water and re-extracted with 2 l of ethyl acetate. Ethyl acetate soluble fraction was evaporated under a vacuum at 45 °C. The extract was subjected to silica gel column chromatography (230—400 mesh, Merck, Darmstadt, Germany). The subfraction that showed the highest inhibitory effect on MMP-1 expression was applied to ODS open column (YMC GEL ODS-AQ, 120A, 50 μm, YMC Co., Ltd., Japan). And the active fraction of 20% methanol

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elution was analyzed on C18 reverse-phase column (ODS aqua, 125A, 250×10 mm, Phenomenix) with HPLC analysis system, and eckol (48 mg) and dieckol (83 mg) were purified.

Luciferase Reporter Gene Assay HeLa cells were seeded into 24 well plates and cultured for 24 h in DMEM with 10% FBS before transfection. AP-1-luciferase or NF-κB-luciferase reporter plasmid DNA (each 0.5 μg: Stratagene) was transfected with Lipofectamin transfection reagent (Invitrogen, Carlsbad, CA, U.S.A.). Twenty-four hours after the transfection, the media were replaced by media with or without the extracts and then stimulated with phorbol myristate acetate (PMA), and cell lysates were collected for the luciferase assay 24 h later. The luciferase activities in the cell lysates were measured according to the manufacturer's recommendation.

Western Blot Analysis Conditioned media were centrifuged at 12000×*g* for 10 min at 4 °C and used for western blot analysis. To determine the amounts of type I procollagen (170—190 kDa) secreted into culture media, equal aliquots of conditioned culture media from an equal number of cells were fractionated by 10% SDS-PAGE, transferred to Hybond ECL membrane. The membrane was blocked for 30 min in TBS containing 0.1% tween 20 (TBS-T) and 5% (w/v) dry skim milk powder, and incubated overnight with monoclonal anti-type I procollagen N-terminal extension peptide (SP1. D8) antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, U.S.A.). The membrane was then washed with TBS-T and incubated for 1 h with a secondary antibody. Bound antibodies were visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, IL, U.S.A.).

RT-PCR Total RNA was extracted using easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology, Sungnam, Korea). Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as a template for each reverse transcriptase (RT)-mediated PCR (RT-PCR) by using the ImProm-II Reverse Transcription System kit (Promega) and Taq polymerase (Solgent, Daejeon, Korea). The primer sets were as follows: MMP-1, forward 5′-CGACTCTAGAAACACAAGAGCAA-GA-3′ and reverse 5′-AAGGTTAGCTTACTGTCACACGCTT-3′, TIMP-1, forward 5′-TCCT-GTTGTTGCTGTGGCTGATAGC-3′ and reverse 5′-CAGGCAAGGTGACGGGACTGGAA-GC-3′, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) , forward 5′-CTCATGACCACAGTCCATGCCATC-3′ and reverse 5′-CTGCTTCACCACCTTCTTGATG-TC-3′. Relative intensities were quantified using a densitometric program (Image Gauge, Fujifilm, Japan).

Statistical Analysis Results were expressed as means± S.D. and the Student's *t*-test was used for the statistical analysis between the control group and the samples. $p<0.05$ was considered as statistically significant.

RESULTS

It has been repeatedly demonstrated that reduction of MMP-1 can be a possible strategy for treating and preventing the clinical manifestations of skin aging.[10—12] Transcriptional regulation of MMP-1 is primarily governed by transcription factor AP-1 and NF-κB.[12—14] To search for active compounds from 29 seaweed extracts which are able to inhibit MMP-1 expression, we used reporter gene assay with reporter plasmids containing AP-1 or NF-κB binding element. As shown in Table 1, the extract from *Ecklonia stolonifera*, Alariaceae, showed 76% and 66.7% inhibitory effect on NF-κB and AP-1 reporter activity, respectively. Interestingly, other 2 species in Alariaceae, *Eisenia bicyclis* and *Ecklonia cava*, also showed strong inhibition of transcriptional activity of both NF-κB and AP-1 reporter activity. These results suggest that seaweeds in Alariaceae have more potent inhibitory effect on NF-κB and AP-1 reporter activity than seaweeds in other family. These inhibitory effects might not be attributable to nonspecific cytotoxicity, because cell viability, which was determined by MTS assay, was not affected by the concentration of the extracts used in this experiment (data not shown).

We next examined the effect of extracts of these 3 species on TNFα-induced expression of MMP-1 in human dermal fibroblasts. Treatment with TNFα led to about 2 fold increase in MMP-1 expression and a subsequent loss of type I procollagen (lanes 1 and 2, Figs. 1A and 1D). However, pretreatment with the extracts attenuated TNFα-induced MMP-1 expression as well as basal expression of MMP-1 (Figs. 1A, B). In addition, a loss of collagen by TNFα was restored by pretreatment with the extracts (Fig. 1D) and the expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), an endogenous MMP inhibitor, was not affected by treatment with these extracts (Figs. 1A, C). These results indicate that components in the extracts might prevent the expression of MMP-1 by TNFα in human dermal fibroblasts, leading to inhibition of collagen degradation by MMP-1. We next determined the dose-dependent effect of the extract from *E. stolonifera*, which showed the most potent inhibitory effect, on MMP-1 and TIMP-1 expression. As expected, TNFα-induced expression of MMP-1 was inhibited by pretreatment with the extract in a dose-dependent manner without alteration of TIMP-1 expression (Figs. 2A, B). These results further support the above observation that the extract from *E. stolonifera* could protect collagen degradation in human skin through the inhibition of MMP-1 expression.

Next, we tried to isolate the active compounds from *E. stolonifera* to inhibit MMP-1 expression in human dermal fibroblasts. Two compounds were purified with solvent extraction, open column chromatography, and reverse phase HPLC, and their structures were analyzed by $^{1}H/^{13}C$-NMR (Lambda 300, Jeol, Japan) and Mass spectrometry (Q STAR Pulsar 1, Applied Biosystems, U.S.A.), which were identified as eckol and dieckol, respectively (Fig. 3A). Thus, we examined the effect of eckol and dieckol on MMP-1 and TIMP-1 expression in human dermal fibroblasts. Treatment with eckol or dieckol caused an attenuation of TNFα-induced expression of MMP-1 as well as of basal expression of MMP-1 (Figs. 3B, C). However, the expression of TIMP-1 was not affected (Figs. 3B, D). In addition, treatment with these compounds led to reduction of both NF-κB and AP-1 reporter gene activity (Fig. 4). These results strongly indicate that eckol and dieckol from *E. stolonifera* are major principles which are responsible for the reduction of MMP-1 expression in human dermal fibroblasts.

Table 1. The Inhibitory Effect of Seaweed Extracts on NF-κB or Ap-1 Reporter Gene Activity[a)]

Code No.	Family name	Specific name	Seaweed extracts[b)]		% Inhibition	
			Used amounts (kg, wet wt)	Extract (g)	NF-κB	AP-1
KD-01	Alariaceae	*Eisenia bicyclis*	0.90	3.04	30	52.4
KD-02	Alariaceae	*Ecklonia cava*	0.38	1.73	43	57.1
KD-03	Alariaceae	*Ecklonia stolonifera*	2.0	5.54	76	66.7
KD-04	Cladophoraceae	*Chaetomorpha moniligera*	1.6	1.17	27.5	ND
KD-05	Corallinaceae	*Corallina pilulifera*	0.95	1.14	22.5	75
KD-06	Cryptonemiaceae	*Pachymeniopsis elliptica*	0.33	0.91	<5.0	<5.0
KD-07	Delesserizceae	*Delesseria serrulata*	0.90	1.30	<5.0	29
KD-08	Delesserizceae	*Acrosorium polyneurum*	0.65	0.64	52	42
KD-09	Desmarestiaceae	*Desmarestia ligulata*	0.70	1.71	42	13
KD-10	Desmarestiaceae	*Desmarastia ligulata*	1.8	3.50	24	ND
KD-11	Dictyotaceae	*Dictyota dichotoma*	0.47	1.68	23	<5.0
KD-12	Gigartinaceae	*Gigartina tenella*	0.34	0.74	34	<5.0
KD-13	Gigartinaceae	*Chondrus ocellatus*	1.97	3.20	<5.0	<5.0
KD-14	Gracilariaceae	*Gracilaria verrucosa*	0.91	1.64	44	<5.0
KD-15	Laminariaceae	*Agarum cribrosum*	1.4	3.31	87	<5.0
KD-16	Laminariaceae	*Laminaria japonica*	1.0	1.87	20	79.3
KD-17	Lomentariaceae	*Lomentaria catenata*	0.22	0.16	32	<5.0
KD-18	Phyllophoraceae	*Gymnogongrus flabelliformis*	0.90	0.94	<5.0	<5.0
KD-19	Plocamiaceae	*Plocamium telfairiae*	1.23	0.47	22	<5.0
KD-20	Rhodomelaceae	*Laurencia pinnata*	1.0	2.00	22.5	78.8
KD-21	Rhodomelaceae	*Polysiphonia morrowii*	1.8	3.79	<5.0	84.6
KD-22	Rhodymeniaceae	*Chrysymenia wrightii*	0.40	0.73	<5.0	9
KD-23	Sargassaceae	*Sargassum confusum*	0.95	2.35	<5.0	<5.0
KD-24	Sargassaceae	*Sargassum horneri*	0.44	2.92	<5.0	<5.0
KD-25	Scytosiphonaceae	*Endarachne binghamiae*	0.44	2.73	79	<5.0
KD-26	Scytosiphonaceae	*Colpomenia bullosa*	1.25	3.30	32.5	80.8
KD-27	Scytosiphonaceae	*Scytosiphona lomentaria*	0.80	0.71	45	62
KD-28	Ulvaceae	*Enteromorpha linza*	0.24	0.23	<5.0	29
KD-29	Zosteraceae	*Zostera marina*	0.90	0.22	<5.0	<5.0

a) HeLa cells were transfected with the NF-κB luciferase or AP-1 luciferase reporter plasmid DNA (each 0.5 μg) for 24 h. After pretreatment with the extracts (each concentration 10 μg/ml) for 30 min, the cells were incubated for additional 24 h in the presence of 50 nM PMA. Cells were harvested, and luciferase activity was determined. Data were expressed as mean from three separate experiments in duplicate. ND, not determined. *b*) Seaweed extracts were prepared according to 'Preparation of Seaweed Extracts' in Materials and Methods.

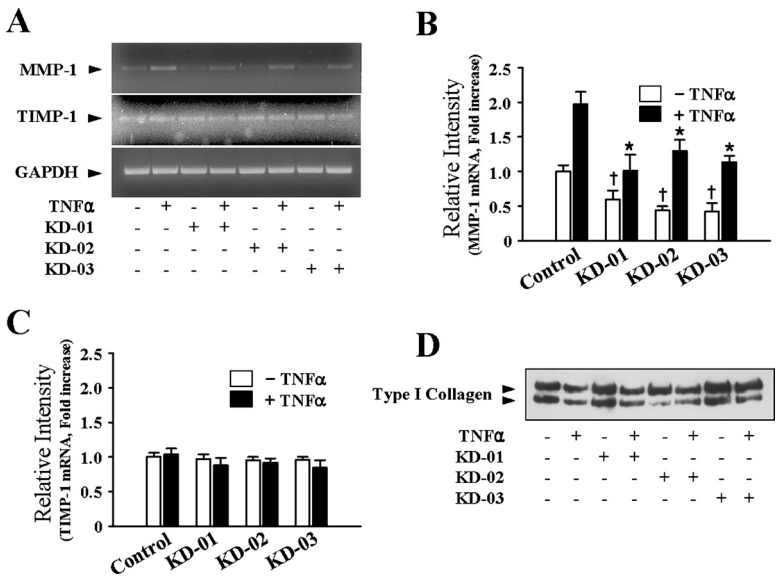

Fig. 1. Three Seaweed Extracts from Alariaceae Inhibit TNFα-Induced Expression of MMP-1 in Human Dermal Fibroblasts

After pretreatment of quiescent human dermal fibroblasts with seaweed extracts (each concentration 10 μg/ml), the cells were harvested 24 h following the addition of TNFα 10 ng/ml. The total RNA from each group was isolated and mRNA levels of MMP-1 and TIMP-1 were analyzed by RT-PCR (A). Relative intensities of MMP-1 (B) and TIMP-1 (C) were quantitated and expressed as mean±S.D. from three independent experiments. †$p<0.05$, control *vs.* sample-treated group; ∗<0.05, TNFα-control *vs.* TNFα/sample-treated group. (D) Conditioned media from the fibroblast culture at 48 h following exposure to TNFα 10 ng/ml were collected, and the level of type I procollagen was examined using immunoblot analysis. KD-01, *E. bicyclis*; KD-02, *E. cava*; KD-03, *E. stolonifera*

DISCUSSION

MMPs, a family of structurally related zinc and calcium-dependent endopeptidases, constitute a large family of proteases that have been identified and classified in more than 20 species.[8] Expression of MMPs is usually elevated by the various extracellular stimuli including growth factors, cytokines, tumor promoters, and UV, and this increase in MMPs seems to be involved in the pathogenesis of diseases such as inflammation and cancer as well as in physiologic processes.[15] In particular, the increased expression of MMPs in dermal fibroblasts is closely associated with skin aging phenotypes such as the skin wrinkling and the loss of elasticity, because increased expression of MMPs during skin aging process leads to a loss of collagen in dermis, resulting from degradation of collagen that is substrate for MMPs, and subsequently leads to destruction of extracellular matrix (ECM).[13,15] Among MMPs reported previously, it has been established that MMP-1 functions as a key enzyme for breakdown of ECM in human skin aging process,[9,10] suggesting that MMP-1 could be a possible target for prevention of skin aging. Indeed, accumulating evidences indicated that an inhibitor of MMP-1 could be a promising agent for the prevention and treatment of skin aging.[10—12] In this study, we demonstrate the inhibitory effect of eckol and dieckol from *Ecklonia* species on MMP-1 expression in human dermal fibroblasts and a possibility to develop as an agent for the prevention and treatment of skin aging.

It has been demonstrated the pivotal role of AP-1 transcription factor in transcriptional regulation of MMP-1 in

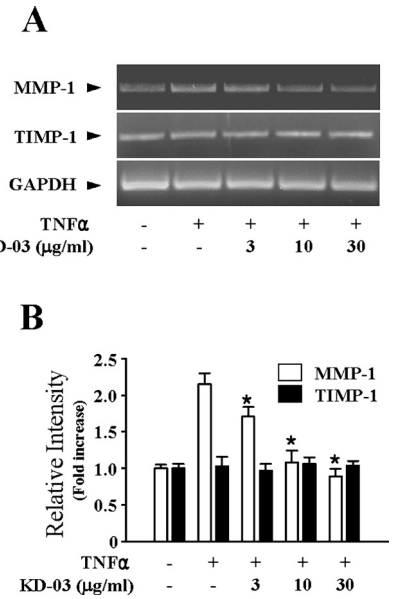

Fig. 2. KD-03 Inhibits TNFα-Induced Expression of MMP-1 in a Dose-Dependent Manner

After pretreatment of quiescent human dermal fibroblasts with KD-03 (*E. stolonifera*), the cells were harvested 24 h following the addition of TNFα 10 ng/ml. The total RNA from each group was isolated and mRNA levels of MMP-1 and TIMP-1 were analyzed by RT-PCR (A). Relative intensities of MMP-1 and TIMP-1 were quantitated and expressed as mean±S.D. from three independent experiments (B). $*<0.05$, TNFα-control *vs.* TNFα/sample-treated group.

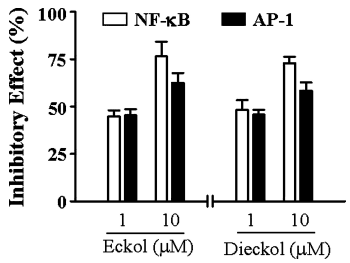

Fig. 4. Eckol and Dieckol Inhibit Both NF-κB and AP-1 Dependent Reporter Gene Activity

HeLa cells were transfected with the NF-κB luciferase or AP-1 luciferase reporter plasmid DNA (each 0.5 μg) for 24 h. After pretreatment with eckol and dieckol for 30 min, the cells were incubated for additional 24 h in the presence of 50 nM PMA. Cells were harvested, and luciferase activity was determined.

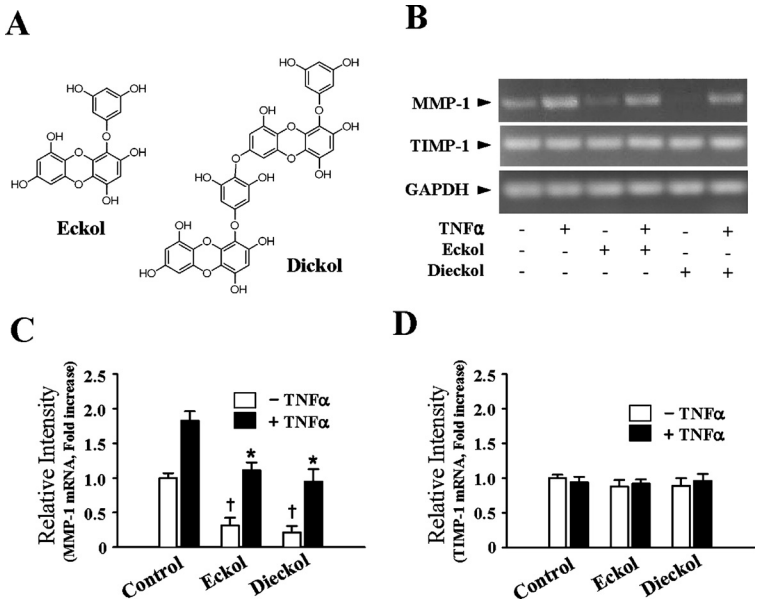

Fig. 3. Eckol and Dieckol Inhibit MMP-1 Expression in Human Dermal Fibroblasts

(A) Structures of eckol and dieckol isolated from *E. stolonifera*. (B) After pretreatment of quiescent human dermal fibroblasts with eckol 10 μM and dieckol 10 μM, the cells were harvested 24 h following the addition of TNFα 10 ng/ml. The total RNA from each group was isolated and mRNA levels of MMP-1 and TIMP-1 were analyzed by RT-PCR. Relative intensities of MMP-1 (C) and TIMP-1 (D) were quantitated and expressed as mean±S.D. from three independent experiments. $\dagger p<0.05$, control *vs.* sample-treated group; $*<0.05$, TNFα-control *vs.* TNFα/sample-treated group.

many cells.[16,17] In addition, accumulating studies have shown that cooperation of AP-1 with NF-κB is necessary for full activation of MMP-1 transcription.[14,18] Therefore, to isolate active components which are able to inhibit MMP-1 expression, we selected the seaweed extracts from 3 species in Alariaceae that have showed strong inhibition of both NF-κB and AP-1 dependent reporter activity, although some extracts have showed selective inhibition of AP-1 or NF-κB (Table 1). Indeed, treatment of human dermal fibroblasts with these extracts led to a decrease in TNFα-induced expression of MMP-1 as well as in basal expression of MMP-1, leading to restoration of collagen contents. In addition, our data suggest that eckol and dieckol are active principles of *Ecklonia* species to inhibit MMP-1 expression, because treatment with eckol or dieckol causes a reduction of MMP-1 expression resulting from the inhibition of NF-κB and AP-1 dependent reporter gene activity. Phlorotannins such as eckol and dieckol were firstly isolated from *Ecklonia* species, which are oligomeric polyphenol of phloroglucinol unit.[19,20] It has been reported that phlorotannins from *Ecklonia* species exhibit free radical scavenging activity,[20,21] anti-plasmin inhibiting activity,[19,22] anti-mutagenic activity,[23,24] bactericidal activity,[25] HIV-1 reverse transcriptase and protease inhibiting activity,[26] and tyrosinase inhibitory activity.[27] These facts imply that phlorotannins such as eckol and dieckol are responsible for these biological activities of *Ecklonia* species. Although at present we do not explain the exact molecular mechanism for inhibition of MMP-1 expression by eckol and dieckol, one possible explanation could be their anti-oxidant activities. Accumulating evidences suggest that various antioxidants such as quercetin, β-carotene, epigallocatechin-3-gallate, and vitamin E inhibit the expression of MMP-1, because reactive oxygen species could play as an important signaling mediator for MMP-1 expression by exogenous stimuli including UV and cytokines.[28—31] In support of this assumption, eckol and dieckol have shown to inhibit the generation of reactive oxygen species (data not shown), and their antioxidant activities were comparable to that of trolox, a well known antioxidant.[20,21]

In conclusion, we identified that the extract from 3 species of Alariaceae could inhibit the expression of MMP-1 in human dermal fibroblast, and that eckol and dieckol from *E. stolonifera* play a major role to inhibit MMP-1 expression. These results suggest the usefulness of eckol and dieckol as a potential preventive or therapeutic agent for skin aging.

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