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Chapter 23

Study of the Anti-Photoaging Effect of Noni (*Morinda citrifolia*)

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Additional information is available at the end of the chapter

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1. Introduction

During the aging process, morphological changes in the human skin appear most noticeably in areas of frequent exposure to ultraviolet (UV) light from the sun, such as the face and hands. Chronic UV exposure induces photoaging, characterized by pigmented spots and wrinkles in the skin. Gradual destruction of the ozonosphere has raised photoaging risk. This has led to rapid growth of the anti-photoaging cosmetic market, especially among women with young and fair skin. Sunscreen agents are a first choice for protection against photoaging. However, a certain amount of UV irradiation penetrates skin dermis, and adverse effect may occur with use of these agents. Because of this, a current trend is the development of safer cosmetic ingredients that effectively inhibit the UV signaling pathways leading to photoaging [1].

Therefore, in this chapter we discuss searching for novel cosmetic ingredients from natural resources which prevent the generation of pigmented spots and wrinkles *via* antagonistic activities against UV signaling pathways. We focused this search on plants growing along the coasts of South Pacific islands, as they may have developed specific self-defense systems against harmful UV radiation. Following this strategy, we selected “noni” (*Morinda citrifolia* L., Rubiaceae).

2. Subtropical plant noni

Selected *M. citrifolia* as a subject is commonly called "noni", which is a subtropical plant distributed widely in the tropical/ subtropical zone, including Tahiti and Hawaii. The tree is a rapidly growing evergreen that is resistant to drought and poor soil conditions.

Noni has been utilized for various reasons in many areas. The root was used as dye in Japan, and the leaves and the seeds, as well as the fruit, were eaten frequently in Southeast Asia and the Pacific islands. Currently, the fruit juice and leaf tea are sold in the functional foods market. Noni is also used as an herbal medicine to promote health and beauty. The whole plant, from the root to fruit, has been used without waste for more than 2000 years as a panacea [2]. Therefore, noni was considered a "gift from God" by generations of Pacific Islanders.

In the last decade, many papers have reported the chemical constituents and biological activities of noni including hypotensive [3], hypoglycemic [4], and anticancer activities [5], which scientifically support traditional claims of noni. Noni has been used traditionally for the treatment of dermatoses such as ringworm, dry skin, acne, pustule, and other skin troubles. Moreover, ripe noni fruit juice has been drunk for cosmetic reasons. In some areas, the dried immature noni fruit, the leaves, or the seeds mixed with coconut oil have been used as an external treatment [6]. But studies reporting potential cosmetic uses of noni, such as inhibition of melanogenesis and reduction of wrinkles, have only recently been completed. Noni fruit contains a large number of seeds throughout its flesh. But during the production of noni fruit juice, the seeds are removed and discarded. Considering the potential utility of all parts of the plant, we investigated extracts from the fruit flesh, leaves, and seeds for active anti-photoaging agents.

3. Skin whitening effect of noni

3.1. Screening tests for melanogenesis inhibitory effect of noni

During photoaging, UV rays trigger melanogenesis and chromatosis. These processes generate pigmented spots by UV activation of melanocyte tyrosinase (a melanin synthesis enzyme) which then converts L-tyrosine to L-DOPA, followed by conversion to dopaquinone. Dopaquinone subsequently forms melanin through several steps, including auto-oxidation. As such, tyrosinase inhibitors may be useful for prevention of pigmented spots.

Initial evaluation of the anti-melanogenesis activity of noni was carried out using an *in vitro* tyrosinase inhibition assay with 50% ethanol extracts of fruit flesh (Fruit-ext), leaves (Leaf-ext), and seeds (Seed-ext). As oxidative reactions also contribute to melanogenesis [7], the 1-diphenyl-2-picrylhydrazyl (DPPH) assay was also performed to find whether noni has antioxidant activity.

3.1.1. Tyrosinase inhibitory activities

The results of the *in vitro* tyrosinase inhibition assay are shown in Table 1. At 20 to 500 μg/ml, Seed-ext inhibited tyrosinase activity, in a concentration-dependent manner. Fruit-ext exhibited weak activity only at 500 μg/ml, and Leaf-ext did not inhibit enzyme activity at any concentration [8].

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3.1.2. DPPH radical scavenging activities

As oxidative reactions contribute to melanogenesis, the DPPH assay was performed to measure the antioxidant activity of noni. As shown in Table 2, Seed-ext exhibited potent DPPH radical scavenging activity, with an IC_{50} value of 12 μg/ml. Leaf-ext and Fruit-ext exhibited weaker antioxidant activities, with IC_{50} values of 113 and 240 μg/ml, respectively [8].

The results of the two assays reveal that Seed-ext has stronger tyrosinase inhibitory and antioxidant activity than Fruit-ext and Leaf-ext.

Samples	Concentration	OD (×1000) [a)] at 475 nm	Inhibition (%)
Control		472±4	
Fruit-ext	20 (μg/ml)	471±3	0
	100 (μg/ml)	451±2	5
	500 (μg/ml)	419±9[i]	11
Leaf-ext	20 (μg/ml)	471±2	0
	100 (μg/ml)	460±2	3
	500 (μg/ml)	442±1[i]	6
Seed-ext	20 (μg/ml)	449±5[i]	5
	100 (μg/ml)	394±4[i]	17
	500 (μg/ml)	365±4[i]	23
Kojic acid	10 (μM)	207±3[i]	56
	50 (μM)	77±3[i]	84

Table 1. Tyrosinase Inhibitory Activities of Fruit-ext, Leaf-ext, Seed-ext and Kojic Acid ([a)] OD: optical density. Each value represents the mean±S.E. of 3 experiments. Significantly different from control group, [i]: $p<0.01$.)

Samples	Concentration	OD (×1000) [a)] at 520 nm	Inhibition (%)	IC_{50} value [b)]
Control		974±21		
Fruit-ext	100 (μg/ml)	759±3[i]	22	240 (μg/ml)
	200 (μg/ml)	536±5[i]	45	
	400 (μg/ml)	188±3[i]	81	
Leaf-ext	50 (μg/ml)	765±4[i]	22	113 (μg/ml)
	100 (μg/ml)	570±3[i]	42	
	200 (μg/ml)	301±6[i]	69	
Seed-ext	5 (μg/ml)	771±2[i]	21	12 (μg/ml)
	10 (μg/ml)	537±7[i]	45	
	20 (μg/ml)	121±5[i]	88	
L-Ascorbic acid	20 (μM)	570±4[i]	41	23 (μM)
	50 (μM)	79±2[i]	92	

Table 2. DPPH Radical Scavenging Activities of Fruit-ext, Leaf-ext, Seed-ext and L-Ascorbic Acid ([a)] OD: optical density. Each value represents the mean±S.E. of 3 experiments. Significantly different from control group, [i]: $p<0.01$. [b)] IC_{50} value represents the concentration of sample required to scavenge 50% of DPPH free radical.)

3.2. Inhibitory effect of noni seeds on melanogenesis and its active compounds

According to the *in vitro* the screenings, Seed-ext may have melanogenesis inhibitory properties. Further examination of Seed-ext involved the use of B16 murine melanoma cells as an *in vitro* melanogenesis test model. In this assay, cells were stimulated by α-melanocyte stimulating hormone (α-MSH) and incubated for 72 hrs with the vehicle or test material [9]. As shown in Table 3, vehicle control treated cells, stimulated with α–MSH, significantly promoted melanogenesis compared to control that was not stimulated with α-MSH. At concentrations ranging from 12.5 to 200 μg/ml, Seed-ext inhibited α-MSH-stimulated melanogenesis in a concentration dependent manner without any significant effects on cell proliferation [10].

The tyrosinase inhibitory activity of Seed-ext was not as potent as that of other well known skin whitening agents. But in the B16 melanoma cells culture system, it inhibited melanin production. Thus, noni seed may be useful as an anti-photoaging cosmetic ingredient which prevents pigmented spots by interacting with a different active site than existing general skin whitening agents.

Samples	Concentration	*α*-MSH (μM)	Melanin content (μg/well)	Cell proliferation (%)
Control			1.6±0.4	61.9±1.2
Vehicle control		1	42.8±1.6[ii]	100.0±1.2[ii]
Seed-ext	12.5 (μg/ml)	1	29.0±0.6[i]	100.6±1.1
	50 (μg/ml)	1	21.1±0.6[i]	105.9±1.2
	200 (μg/ml)	1	11.9±0.6[i]	103.5±2.5
Kojic acid	100 (μM)	1	13.5±0.4[i]	107.4±1.9
	200 (μM)	1	6.9±0.4[i]	103.2±2.4

Table 3. Effects of Seed-ext and Kojic Acid on α-MSH-Stimulated Melanogenesis in B16 Melanoma Cells (Each value in melanin content represents the mean±S.E. of 3 experiments. Significantly different from the control group, [ii]: $p<0.01$. Significantly different from the vehicle control group, [i]: $p<0.01$. Each value in cell proliferation represents the mean ±S.E. of 3 experiments.)

As Seed-ext was confirmed to potently inhibit melanogenesis, activity guided isolation of the active compounds was carried out. Two lignans, 3,3'-bisdemethylpinoresinol (**1**) and americanin A (**2**), were isolated from noni seeds and found to be active constituents. As shown in Table 4, 10 and 20 μM of **1** displayed weak inhibition of cell proliferation. But 1.25 to 5 μM of **1** inhibited melanogenesis in a concentration dependent without any significant effects on cell proliferation. Also, 100 and 200 μM of **2** inhibited melanogenesis [10]. **1** (IC_{50} value: 0.3 mM) and **2** (IC_{50} value: 2.7 mM) exhibited tyrosinase inhibition, and **1** (IC_{50} value: 4 μM) and **2** (IC_{50} value: 11 μM) exhibited potent DPPH radical scavenging properties [8].

Samples	Concentration (μM)	α-MSH (μM)	Melanin content (μg/well)	Cell proliferation (%)
Run 1				
Control			4.5±0.8	55.2±1.3
Vehicle control		1	37.5±1.5[ii]	100.0±1.1[ii]
1	1.25	1	29.9±0.6[i]	97.8±0.4
	2.5	1	21.6±0.8[i]	101.0±1.7
	5	1	18.5±1.2[i]	99.1±0.7
	10	1	15.2±0.2[i]	88.8±1.2[i]
	20	1	13.2±0.8[i]	88.5±0.3[i]
Kojic acid	100	1	17.2±1.1[i]	97.1±1.3
	200	1	6.4±0.4[i]	98.2±1.4
Run 2				
Control			7.3±2.5	58.8±2.4
Vehicle control		1	39.0±0.9[ii]	100.0±5.1[ii]
2	12.5	1	39.3±0.7	101.6±1.0
	25	1	36.7±0.7	107.1±0.4
	50	1	33.9±0.8	103.3±0.9
	100	1	25.5±1.5[i]	99.4±4.5
	200	1	13.9±1.0[i]	102.8±1.4
Kojic acid	100	1	15.7±1.0[i]	101.9±3.8
	200	1	8.6±0.6[i]	98.4±2.7

Table 4. Effects of 3,3′-Bisdemethylpinoresinol (1), Americanin A (2) and Kojic Acid on α-MSH-Stimulated Melanogenesis in B16 Melanoma Cells (Each value in melanin content represents the mean±S.E. of 3 experiments. Significantly different from the control group, [ii]: $p<0.01$. Significantly different from the vehicle control group, [i]: $p<0.01$. Each value in cell proliferation represents the mean±S.E. of 3 experiments.)

Compared to kojic acid, which is a common skin whitening ingredient, **1** exhibited more potent inhibition of melanogenesis. This suggested that the anti-melanogenesis effect of **1** may be due to the suppression of tyrosinase protein expression in the cells, rather than inhibiting tyrosinase itself.

3.3. Effects of noni compounds; 3,3'-bisdemethylpinoresinol and americanin A on inhibitory melanogenesis activity

As the anti-melanogenesis of Seed-ext may be due to **1** and **2**, the inhibitory mechanism of these two lignans was studied with α-MSH stimulated B16 melanoma cells.

3.3.1. Effects of 3,3′-bisdemethylpinoresinol and americanin A on tyrosinase expression in B16 melanoma cells

First, the effect of **1** and **2** on tyrosinase expression in α-MSH stimulated B16 melanoma cells was investigated by using Western blot analysis. As shown in Fig. 1, the tyrosinase expression of the control at 72 hrs was enhanced remarkably. But after 72 hrs of treatment with **1** (5 μM) or **2** (200 μM), the enhancement of expression was notably suppressed without any significant effect on cell proliferation [10].

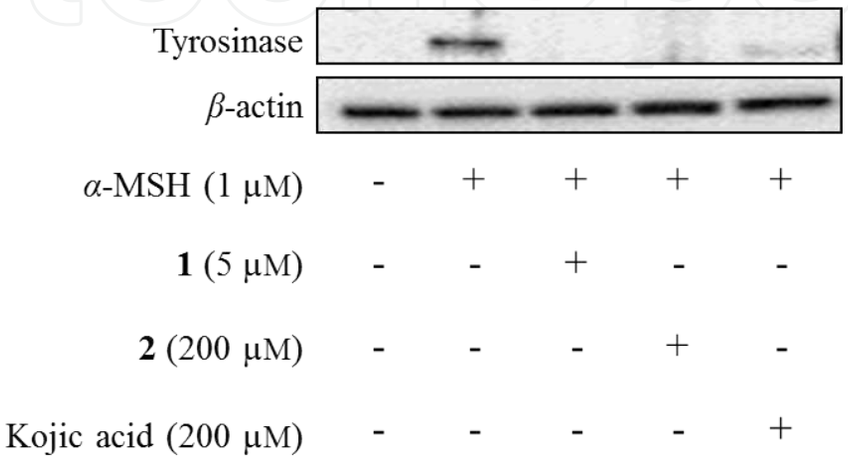

Figure 1. Effects of 3,3′-Bisdemethylpinoresinol (1), Americanin A (2) and Kojic Acid on Tyrosinase Expression in α-MSH-Stimulated B16 Melanoma Cells (The cells were treated with α-MSH (1 μM) in the presence of **1** (5 μM), **2** (200 μM) or kojic acid (200 μM) for 72 hrs. The level of tyrosinase expression was examined by Western blot analysis using specific antibody. Equal protein loading was confirmed by β-actin expression.)

3.3.2. Inhibition of tyrosinase in B16 melanoma cells by 3,3′-bisdemethyl-pinoresinol and americanin A

With suppressed expression of the enzyme, the activity of tyrosinase in the cell may decrease. Secondly, α-MSH stimulated B16 melanoma cells were cultivated during treatment with **1** or **2**. Next, the amount of melanin and tyrosinase activity in the cells were measured.

As shown in Fig. 2A, the intracellular melanin content of control group increased remarkably after cultivation for 24 to 72 hrs, whereas the content in cells treated with **1** (5 μM) or **2** (200 μM) decreased after 72 hrs. As shown in Fig. 2B, the tyrosinase activity in the α-MSH stimulated cells was also significantly inhibited by addition of **1** (5 μM) or **2** (200 μM) after 24 to 72 hrs incubation [10].

As just described, lignans **1** (5 μM) and **2** (200 μM) inhibited intracellular melanin contents induced by α-MSH. The results of Western blot analysis demonstrated that **1** and **2** remarkably inhibit tyrosinase stimulated by α-MSH. Moreover, as **1** and **2** reduced intracellular tyrosinase activity, it became clear that their melanogenesis inhibitory activity involved suppression of tyrosinase expression.

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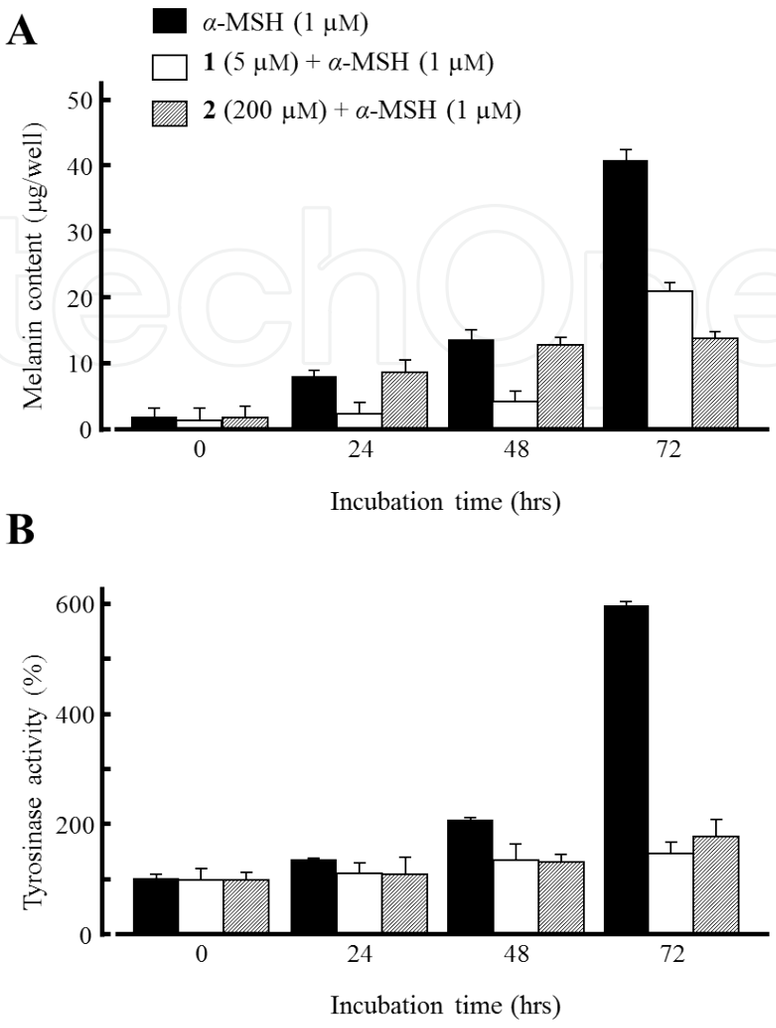

Figure 2. Effects of 3,3′-Bisdemethylpinoresinol (1) and Americanin A (2) on Melanogenesis and Tyrosinase Activity in α-MSH-Stimulated B16 Melanoma Cells (The cells were treated with α-MSH (1 μM, black column), **1** (5 μM, white column) and **2** (200 μM, slashed column) for the indicated times. (A) The melanin content was determined. (B) Tyrosinase activity was determined by measuring the formation of dopachrome. Data represent mean±S.E. of two different experiments each carried out in triplicate.)

3.3.3. Effect of 3,3′-bisdemethylpinoresinol and americanin A on phosphorylation of p38 MAPK

During melanogenesis in melanocytes, it is known that microphthalmia-associated transcription (MITF) is a transcription factor that regulates expression of the tyrosinase gene, and that melanins are produced by the activation of MITF [11]. Since mitogen-activated protein kinases (MAPKs) pathway is one of the intracellular signals that activates MITF, further research on this pathway was performed. It has been reported that phosphorylation of p38 MAPK activates MITF, whereas that of ERK1/2 and p70 S6K suppress MITF [12, 13]. The effects of **1** and **2** on the MAPK signaling activities of α-MSH-stimulated B16 melanoma cells were examined.

The levels of phosphorylation of p38 MAPK were compared, at 6 and 12 hrs after stimulation of B16 melanoma cells by α-MSH (1 μM). As shown in Fig. 3A, the analysis at 6 and 12 hrs reveals that the levels of phosphorylation of p38 MAPK in B16 cells were enhanced by α-MSH treatment in comparison to those without α-MSH. The treatment with lignan **1** (5 μM) or lignan **2** (200 μM) suppressed phosphorylation of p38 MAPK and enhanced that of ERK1/2 at 6 and 12 hrs after stimulated by α-MSH (Fig. 3B). However, both lignans had no effect on p70 S6K phosophorylation (Fig. 3B) [10].

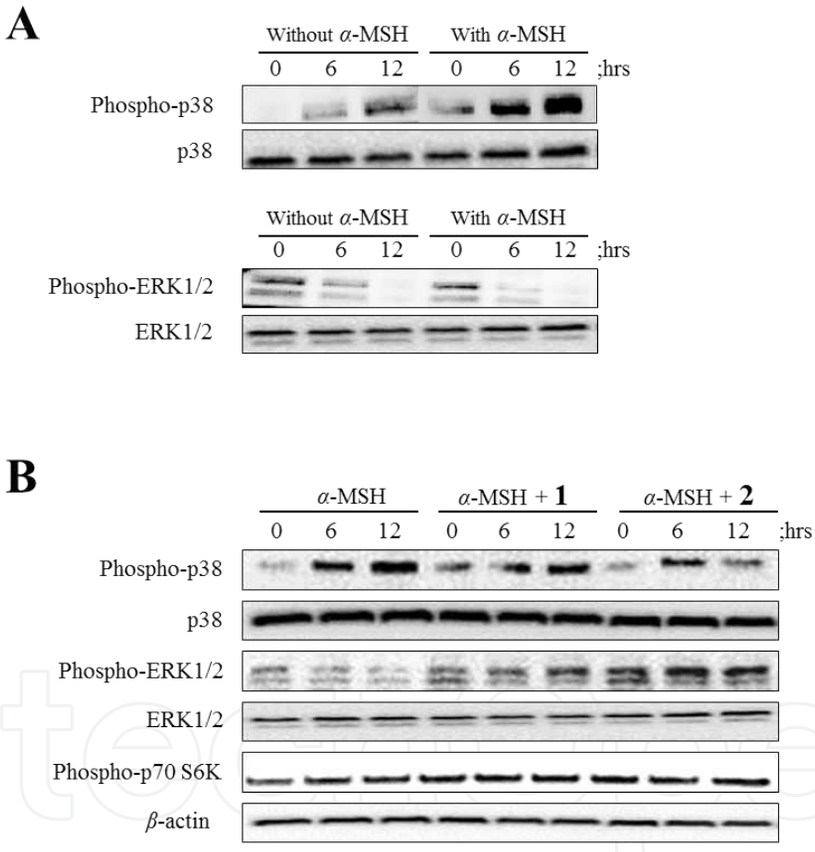

Figure 3. (A) Effect of α-MSH on Phosphorylation of p38 MAPK and ERK1/2 in B16 Melanoma Cells. (B) Effects of 3,3′-Bisdemethylpinoresinol (1) and Americanin A (2) on Phosphorylation of p38 MAPK, ERK1/2 and p70 S6K in α-MSH-Stimulated B16 Melanoma Cells ((A) The cells were treated with or without α-MSH (1 μM) for the indicated times. (B) The cells were treated with α-MSH (1 μM) in the presence of **1** (5 μM) or **2** (200 μM) for the indicated times. Phosphorylation of p38 MAPK, ERK1/2, and p70 S6K was assessed by Western blot analysis with using specific antibody for phosphorylated forms of p38 MAPK, ERK1/2, and p70 S6K. Equal protein loading was confirmed by β-actin expression.)

As the inhibitory mechanism of melanogenesis in α-MSH-stimulated B16 melanoma cells, These results strongly suggest that the lignans inhibit tyrosinase expression by suppressing p38 MAPK phosphorylation and enhancing ERK1/2, which then prevents activation of MITF.

4. Inhibitory effect of noni on wrinkle formation

It is thought that wrinkles form mainly by aging, but they are also caused by photoaging. The causes are degradation of moisture-retaining property of epidermis and transformation of dermal configuration due to altered elastic or collagen fibers in the cutis. Anti-wrinkle cosmetics are useful for comparatively slight wrinkles and fine lines. The main functions of anti-wrinkle cosmetics included normalization of moisture retention by the cornified layer, acceleration of keratinocyte turnover, and acceleration of collagen and elastin synthesis through proliferation and stimulation of fibroblasts. Recently, retinol and its analogs, so-called retinoids, have received increased attention and utilization as a cosmetic ingredient because they promote fibroblastic collagen production in the cutis and improve skin tension. Also, vitamin A reportedly accelerates turnover, which reduces hyperpigmented skin spots caused by melanin excreted by the epidermis, even though it has no inhibitory effects on activation of tyrosinase and melanogenesis. But retinoids irritate the skin and have adverse effects such as dermatitis. Therefore, safer reducing wrinkle ingredients are needed [1].

4.1. Wrinkle inhibition screening test of noni

One cause of photoaging wrinkle formation is the degradation of collagen, a main component of corium connective tissue [14]. The degradation of collagen is promoted by release of human leukocyte elastase (HLE) from infiltrated neutrophils into the skin by UV irradiation [15]. HLE cleaves the triple helix structure of type I collagen and degrades elastic fiber in human skin [16]. Thus, HLE inhibitors may be useful for the prevention of wrinkle formation.

4.1.1. Inhibitory effect of noni on HLE and its active compounds

The inhibitory effect of noni on wrinkle formation was investigated by measuring HLE inhibiting activity *in vitro*. As shown in Table 5, concentrations raging from 0.5 to 1.0 mg/ml of Seed-ext displayed HLE inhibitory activity in a concentration-dependent manner. Fruit-ext and Leaf-ext had no inhibitory activity at 1.0 mg/ml [8].

As Seed-ext exhibited potent HLE inhibitory activity compared with Fruit-ext and Leaf-ext, noni seeds appear to be the most source of cosmetic ingredients capable of preventing wrinkle formation during photoaging.

The active compounds in Seed-ext were isolated using the HLE inhibition bioassay as a fractionation guide. Ursolic acid (**3**) was isolated from Seed-ext and found to the active constituent. The IC_{50} value of **3** in the HLE inhibition assay was 0.07 mM. The IC_{50} value of phenylmethanesulfonyl fluoride (PMSF), the positive control, was 0.14 mM. Thus, **3** was more potent than the positive control (data not shown) [8].

Samples	Concentration	OD (×1000) [a)] at 405 nm	Inhibition (%)	IC_{50} value [b)]
Control A [c)]		946±23		
Fruit-ext	0.1 (mg/ml)	994±38[i]	-8	
	0.5 (mg/ml)	1014±9[i]	-10	
	1.0 (mg/ml)	1039±9[i]	-12	
Leaf-ext	0.1 (mg/ml)	1014±16[i]	-10	
	0.5 (mg/ml)	1023±18[i]	-11	
	1.0 (mg/ml)	1042±36[i]	-13	
Seed-ext	0.1 (mg/ml)	1052±20[i]	-14	1.0 (mg/ml)
	0.5 (mg/ml)	722±21[i]	22	
	1.0 (mg/ml)	467±30[i]	50	
Control B [d)]		925±9		
PMSF	0.08 (mM)	676±7[ii]	29	0.14 (mM)
	0.15 (mM)	383±14[ii]	60	
	0.50 (mM)	100±1[ii]	90	

Table 5. HLE Inhibitory Activities of Fruit-ext, Leaf-ext, Seed-ext and PMSF ([a)] OD: optical density. [b)] IC_{50} value represents the concentration of sample required to inhibit 50% of HLE activity. [c)] Control A is a control for extracts. [d)] Control B is a control for PMSF. Each value represents the mean±S.E. of 3 experiments. Significantly different from control A group, [i]: $p<0.01$. Significantly different from control B group, [ii]: $p<0.01$.)

4.2. Inhibitory effect of noni seeds on matrix metalloproteinase-1 (MMP-1) secretion and its active compounds

Matrix metalloproteinases (MMPs) are matrix degrading enzymes associated with destructive processes including inflammation, tumor invasion and skin aging [1]. More than 20 subtypes of MMPs have been reported [17]. Among these, MMP-1, secreted from human skin fibroblasts, is mainly responsible for the degradation of dermal type I collagen in the photoaging process [18]. Also, HLE activates MMP-1 [19]. Thus, it is expected that MMP-1 and HLE inhibitors may be useful for the prevention of photoaging and subsequent wrinkle formation.

Since Seed-ext displayed strong HLE inhibitory activity, its ability to inhibit MMP-1 secretion was investigated in UV-irradiated normal human dermal fibroblasts (NHDFs). The amount of MMP-1 protein secreted from NHDFs into culture media was analyzed by Western blotting. UVA irradiation (5 J/cm^2) enhanced the secretion of MMP-1 from NHDFs in the vehicle control group at 9 to 48 hrs incubation, as compared to the control group without UVA-irradiation (Fig. 4). The group which was treated with Seed-ext (10 μg/ml) after UVA-irradiated NHDFs inhibited the secretion MMP-1 at 24 to 48 hrs, when compared to the vehicle control (Fig. 4) [20]. Seed-ext was not cytotoxic at 3 to 30 μg/ml.

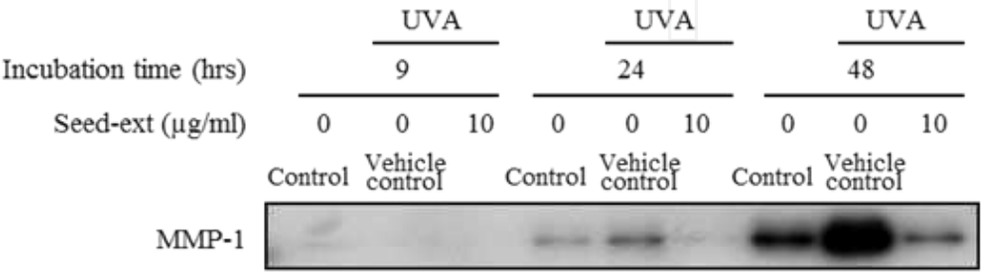

Figure 4. Effect of Seed-ext on MMP-1 Secretion from UVA-Irradiated NHDFs (Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium containing test samples for 9, 24, and 48 hrs. MMP-1 protein levels in the cultured medium at the indicated times were assessed by Western blot analysis using the antibody against human MMP-1. The blot is representative of three separate experiments and represents a single immunoblot.)

The active compounds responsible for the inhibitory effect of Seed-ext on MMP-1 secretion were searched for by following bioassay guided fractionation. As shown in Fig. 5A and 5B, 3,3'-bisdemethylpinoresinol (**1**), which is an active anti-melanogenesis compound, significantly inhibited the secretion MMP-1 at 3 μM. On the other hand, ursolic acid (**3**), which is an active anti-HLE compound, had no effect on MMP-1 secretion (data not shown). There was no cytotoxicity from **1** and **3** at 0.03, 0.1 and 0.3 μM [20].

It is clear that ursolic acid (**3**) inhibits HLE, whereas 3,3'-bisdemethylpinoresinol (**1**) inhibits MMP-1 secretion. Further, Seed-ext inhibits both MMP-1 secretion and HLE activity. There are very few plant extracts or compounds that have both inhibitory effects. Thus, noni seeds may be an ideal cosmetic ingredient to prevent wrinkle formation.

4.3. Inhibitory effect of 3,3′-bisdemethylpinoresinol on MMP-1 secretion

In UV irradiated skin, MMP-1 is a major collagenolytic enzyme responsible for collagen damage [21]. It has been reported that UV irradiation promotes expression of MMP-1 in NHDFs [22] and secretion into culture media [23]. It is also known that UV irradiation activates intracellular fibroblast signals, c-Jun-N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) cascades, promotes phosphorylation of JNK and p38, enhances expression of c-Jun and c-Fos, followed by activation of activation protein-1 (AP-1), and, in the end, enhances expression of MMP-1 [14, 24]. In order to find the mechanism of inhibition of MMP-1, compound **1** was investigated for effects on intracellular MMP-1 expression and activation of MAPKs in UVA-irradiated NHDFs.

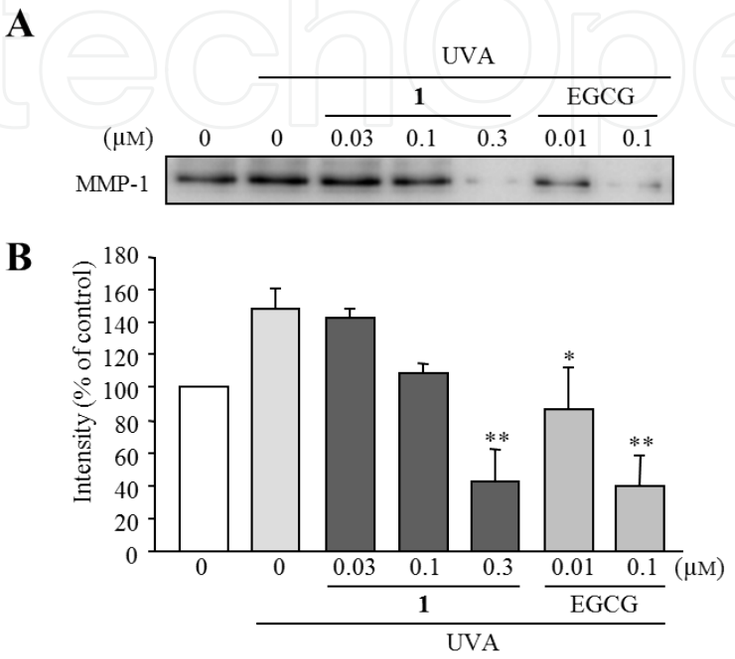

Figure 5. Effects of 3,3′-Bisdemethylpinoresinol (1) and and epigallocatechin-3-*O*-gallate (EGCG) on MMP-1 Secretion from UVA-Irradiated NHDFs (Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium containing test samples for 48 hrs. MMP-1 protein levels in the cultured medium were assessed by Western blot analysis using human MMP-1 specific antibody. (A) The blot is representative of three separate experiments and represents a single immunoblot. (B) All data is reported as mean±S.E. of three separate experiments. Significantly different from the vehicle control group, *: $p<0.05$, **: $p<0.01$.)

4.3.1. Effect of 3,3′-bisdemethylpinoresinol (1) on intracellular MMP-1 expression in UVA irradiation NHDFs

First, the effect of **1** on intracellular MMP-1 expression in UVA-irradiation was examined. In the vehicle control group, UVA irradiation enhanced the levels of intracellular MMP-1 expression at 24 and 48 hrs, with most of the MMP-1 expression being detected at 48 hrs (Fig. 6). But in the treatment group (addition of **1**, 0.3 μM), the expression was down-regulated at 48 hrs (Fig. 6A). The levels of MMP-1 secretion from UVA-irradiated NHDF into the culture medium increased at 48 hrs in the vehicle control group, whereas the treatment group (addition of **1**, 0.3 μM) obviously inhibited MMP-1 secretion (Fig. 6B) [20].

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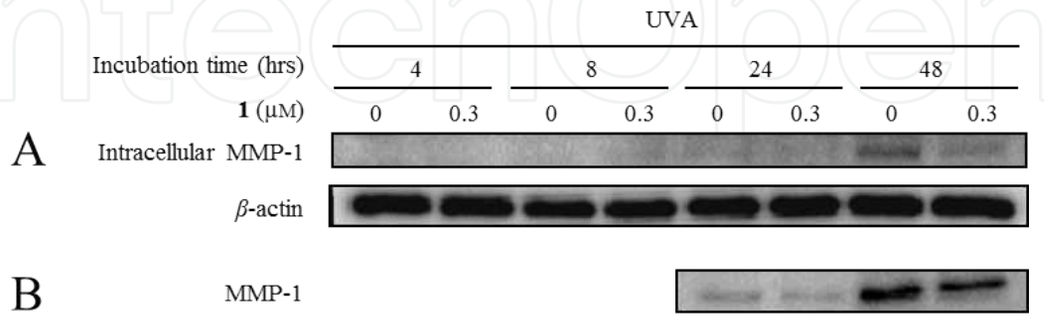

Figure 6. (A) Effect of 3,3′-Bisdemethylpinoresinol (1) on the Intracellular MMP-1 Expression in UVA-Irradiated NHDFs (B) Effect of 3,3′-Bisdemethylpinoresinol (1) on MMP-1 Secretion from UVA-Irradiated NHDFs ((A) Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium containing test samples for 4, 8, 24, and 48 hrs. Intracellular MMP-1 protein levels, at the indicated times, were assessed by Western blot analysis using human MMP-1 specific antibody. The blot is representative of two separate experiments and represents a single immunoblot. (B) Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in serum free medium containing test samples for 24 to 48 hrs. MMP-1 protein levels in cultured medium were assessed by Western blot analysis using human MMP-1 specific antibody. The blot is representative of two separate experiments and represents a single immunoblot.)

4.3.2. Effect of 3,3′-bisdemethylpinoresinol on MAPKs phosphorylation in UVA-irradiated NHDFs

The effect of **1** on activation of MAPKs was investigated by Western blot analysis. JNK and p38 MAPK phosophorylation in UVA irradiated NHDFs versus incubation time is shown in Fig. 7A. JNK phosophorylation was enhanced in a more rapid and transient manner at 0.5 to 1 hr after UVA irradiation, whereas that of p38 MAPK was enhanced at 0 to 1 hr. Therefore, the effect of **1** (0.1 and 0.3 μM) on JNK and p38 MAPK phosophorylation in UVA-irradiated NHDFs was examined at 0.5 hr after irradiation. As shown in Fig. 7B, **1** (0.1 μM) had no inhibitory effect of JNK and p38 MAPK, whereas **1** (0.3 μM) inhibited phosphorylation of both [20].

The results suggest that 1 inhibits MMP-1 secretion in UVA-irradiated NHDFs by decreasing JNK and p38 phosphorylation and suppressing c-Jun and c-Fos expression. This, subsequently, inhibits AP-1 and MMP-1 expression, resulting in a reduction in MMP- secretion.

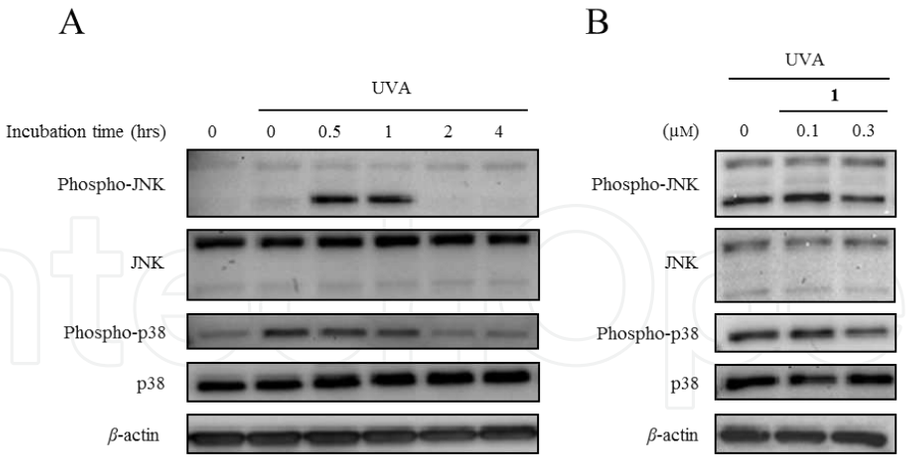

Figure 7. Time Course of MAPKs Phosphorylation in UVA-Irradiated NHDFs (B) Effect of 3,3′-Bisdemethylpinoresinol (1) on MAPKs Phosphorylation in UVA-Irradiated NHDFs ((A) Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium for the indicated time. Total cellular proteins were prepared for Western blot analysis of MAPKs and phospho-MAPKs proteins using the antibodies against phospho-form and total-form of JNK and p38. β-actin was used as an internal control. The blot is representative of three separate experiments and represents a single immunoblot. (B) Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium for 0.5 hr. Total cellular proteins were prepared for Western blot analysis of MAPKs and phospho-MAPKs proteins using the antibodies against phospho-form and total-form of JNK and p38. β-actin was used for internal control. The blot is representative of three separate experiments and represents a single immunoblot.)

5. Inhibitory effect of noni seeds on poor blood fluidity

In addition to melanogenesis and degradation of collagen by HLE and MMP-1, blood stagnation may also contribute to hyperpigmented spots on the skin. In oriental medicine, the clinical state of blood stasis in the venous system is called "Oketsu" in Japanese. Accumulation of wastes caused by blood stagnation in the skin leads to pigmentation. Degradation of blood flow, leading to dry skin and atrophy, also leads to wrinkle formation. Therefore, improved blood fluidity is likely to prevent formation of pigmented spots and wrinkles. As cosmetic ingredients that improve blood fluidity are desired for use in anti-photoaging products, the effect of noni seeds on poor blood fluidity was investigated.

5.1. Antithrombotic effect of noni seeds in a disseminated intravascular coagulation (DIC) rat model

The effect of noni seeds on excessive increased blood coagulation, accompanying poor blood fluidity, was examined by using a DIC rat model, induced by lipopolysaccharide (LPS) [25, 26].

Each Seed-ext (200 and 500 mg/kg) was administrated orally once a day to the rats for 7 successive days. LPS was injected on Day 7. Four hrs after injection, blood samples were collected from the abdominal great vein (vena cava). Whole blood passage time was measured by a micro channel array flow analyzer (MC-FAN). As shown in Table 6, the vehicle control

group (LPS-induced) significantly prolonged blood passage time, in comparison with the control group. However, Seed-ext dose-dependently reduced passage time [27].

Platelet count, fibrinogen and fibrin degradation products (FDP) were also measured. Seed-ext had no effect on any of these hematological parameters (Table 6) [27].

Samples	Dose [a)]	Route [b)]	Blood passage time (s) [c)]	Platelets ($\times 10^4$/μl)	Fibrinogen (mg/dl)	FDP (μg/ml)
Control		*p.o.*	17±1	81.1±2.5	198.0±5.2	0.43±0.06
Vehicle control		*p.o.*	1813±34[ii]	18.4±1.1[ii]	96.6±3.9[ii]	2.06±0.17[ii]
Seed-ext	200 (mg/kg)	*p.o.*	1722±16[iii]	26.7±0.5[i]	96.7±2.9	1.86±0.11
	500 (mg/kg)	*p.o.*	1413±19[i]	22.5±0.8	105.2±2.7	1.75±0.07
Heparin	500 (U/kg)	i.v.	26±1[i]	46.1±2.8[i]	194.1±7.2[i]	0.52±0.15[iii]

Table 6. Effects of Seed-ext and Heparin on Blood Passage Time, Platelet Count, Fibrinogen, and FDP in Rats, after LPS Injection (Each value represents the mean±S.E. (n=7). Significantly different from the control group, [ii]: p<0.01. Significantly different from the vehicle control group, [iii]: p<0.05, [i]: p<0.01. [a)] For 7 successive days, 0.2% CMC-Na was administered orally to control and vehicle control groups. Each extract (200 and 500 mg/kg) was suspended with CMC-Na and administrated orally to each test group once daily for 7 successive days (Day 1-7). 1 hr after the final daily dose on Day 7, LPS (1 mg/kg, dissolved in saline, i.v.) was injected into the tail vein. Heparin (500 U/kg, dissolved in saline, i.v.) was administered intravenously to the rats 1 hr before LPS injection on Day 7 to the heparin group. [b)] *p.o.*: oral administration, i.v.: intravenous administration [c)] Blood passage time measured by MC-FAN as the time taken for the flow of 50 μl of sample mixture (1.8 ml of blood and 0.2 ml of 3.8% sodium citrate solution).

The results from the DIC rat model experiments suggest that Seed-ext improves poor blood fluidity. Therefore, noni seeds may help reduce pigmentation associated with blood stagnation.

5.2. Inhibitory effect of noni seeds, and active consituents, on platelet aggregation

Blood fluidity, as measured by using MC-FAN, is influenced by erythroid deformability, leukocytic adherence ability and thrombocytic agglutinability [28, 29]. Blood flow regulating factors in microcirculation have vascular systemic and blood component functions. Circulatory system function deteriorates with platelet aggregation induced coagulation, degradation of erythrocyte deformability, hemagglutination, elevation of plasma viscosity, and by degradation of fibrinolytic system activation [28, 29].

In order to examine the inhibitory effect of Seed-ext on blood coagulation, collagen-induced platelet aggregation [30] and polybrene-induced erythrocyte aggregation [31], *in vitro* tests were carried out. Seed-ext did not inhibit platelet aggregation (data not shown). But it did inhibit hemagglutination at concentrations ranging from 50 to 500 μg/ml (Table 7). As

shown in Table 7, **3** inhibited platelet aggregation at 10 to 50 μM. Lingnans **1** and **2** had weak effects of platelet aggregation, when comparison to **3** [27].

The results suggest that one inhibitory mechanisms behind the effect of Seed-ext on poor blood fluidity in the DIC rat model may be anti-hemaggulatination by 1, 2, and 3.

Samples	Concentration	Aggregation (%)	Inhibition (%)
Control		45±1	
Seed-ext	50 (μg/ml)	27±1[i]	40
	200 (μg/ml)	8±1[i]	82
	500 (μg/ml)	9±1[i]	81
1	10 (μM)	42±1	7
	20 (μM)	39±1	13
	50 (μM)	34±1[i]	24
	100 (μM)	27±1[i]	41
2	10 (μM)	42±2	7
	20 (μM)	41±1	9
	50 (μM)	38±1	16
	100 (μM)	32±1[i]	29
3	5 (μM)	40±0	11
	10 (μM)	31±2[i]	31
	20 (μM)	10±5[i]	78
	50 (μM)	7±2[i]	84
Neuraminidase	7.8 (mU/ml)	36±2	19
	15.6 (mU/ml)	33±3[i]	27
	31.3 (mU/ml)	23±1[i]	48
	62.5 (mU/ml)	17±1[i]	63
	125 (mU/ml)	8±0[i]	82

Table 7. Effects of Seed-ext, **1**, **2**, **3** and Neuraminidase on Polybrene-Induced Erythrocyte Aggregation (Each value represents the mean±S.E. of 3 experiments. Significantly different from control group, [iii]: $p<0.05$, [i]: $p<0.01$.)

5.3. Fibrinolytic activity of noni seeds in rats

Activation of the fibrinolytic system improves blood flow by promoting the lysis of thrombi in blood vessel walls. To better understand the fibrinolytic potential of Seed-ext, as it relates to degradation of blood fluidity, the euglobulin lysis time (ELT) assay in normal rats was

conducted. ELT is the time required for the disappearance of a fibrin clot produced by the addition of thrombin to the eugloblin fraction obtained from blood samples [32]. A reduction in ELT reveals activation of fibrinolysis activity, whereas an extension in ELT implies reduced activity [32].

Seed-ext was administrated orally, and 1 hr later, blood samples were collected. Then ELT was measured using eugloblin fractions from the sample. As shown in Table 8, Seed-ext significantly reduced ELT at dosages from 50 and 200 mg/kg in dose-dependent manner. This reveals that Seed-ext may have an enhancing effect on fibrinolysis activity [27].

Samples	Dose	Route	ELT (min)
Control A [a)]		*p.o.*	98±2
Seed-ext	50 (mg/kg)	*p.o.*	55±5[i]
	200 (mg/kg)	*p.o.*	42±7[i]
Control B [b)]		i.v.	97±2
Dextran sulphate sodium salt	5 (mg/kg)	i.v.	32±3[ii]

Table 8. Effects of Seed-ext and Dextran Sulphate Sodium Salt on ELT in Rats ([a)] Control A is a control for extracts. [b)] Control B is a control for dextran sulphate sodium salt. Each value represents the mean±S.E. of 7 rats. Significantly different from control A group, [i]: $p<0.01$. Significantly different from control B group, [ii]: $p<0.01$.)

Seed-ext has an inhibitory effect on hemagglutination. But it also activates fibrinolysis, suggesting that it may improve blood flow through anti-coagulation and fibrinolysis systems. As such, noni seeds may be a useful supplementary ingredient for the prevention of both pigmented spots and wrinkles caused by venous blood stagnation.

6. Conclusion

We are the first to investigate and find 4 inhibitory effects—namely tyrosinase, melanogenesis, HLE, and MMP-1—for noni seeds related to prevention of pigmented spots and wrinkles by photoaging. As a desirable anti-photoaging agent that is antagonistic to the UV signaling pathways of photoaging, Seed-ext may be a useful novel cosmetic ingredient for the prevention or treatment for pigmented spots and wrinkles. Since we found Seed-ext may improve blood fluidity, it may also be a useful supplemental ingredient aimed for beauty. However, further research, including clinical trials, is needed.

Noni fruit flesh and leaves have been used as functional foods, but the seeds have been discarded without utilization in most cases. Production of a cosmetic ingredient from noni seeds adds significant value to this largely unused natural resource.

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