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PROTECTIVE EFFECT OF VerbasNol™ ON PHOTOAGING

CONTENTS

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Objective

The purpose of this study is to evaluate the effect of VerbasNol™ on skin anti-aging in normal and UVA and UVB damaged skin. Specifically, the effect of VerbasNol™ on cell proliferation in normal and UVA and UVB damaged skin HDF cells and the intracellular and secreted extracellular collagen 1 and MMP 1 expression in normal and UVA and UVB damaged skin HDF cells were studied.

Introduction

UV irradiation induces photo damage to the skin tissues, which is characterized by distinct alterations in the composition of the dermal extracellular matrix (ECM), resulting in wrinkles, laxity, coarseness, mottled pigmentation and histological changes that include increased epidermal thickness and connective tissue alteration.

Collagen is one of the main components of human skin. It is synthesized in fibroblasts in the form of procollagen which is derived from dermal fibroblasts. The synthesis of collagen is significantly regulated by a variety of cellular factors including cytokines, hormones, and growth factors. Many studies revealed that the level of tissue collagen is degraded primarily by matrix metalloproteinases (MMPs). Studies indicated that the level of MMPs is significantly induced in UV-irradiated cells through the activation of transcription factor kB (NF-kB) and activator protein1 (AP1). The activation and translocation of NF-kB into nuclear stimulate the expression of inflammation cytokines and MMPs in human skin cells. Thus, development of potential agent with efficacy in blocking the expression of MMPs and inflammatory cytokines is vital for protecting against UV-induced damage and anti-aging.

VerbasNol™ is the trademark name of NuLiv's verbascoside, developed exclusively by NuLiv Science. Verbascoside is an extensively studied polyphenolic compound functions to protect the plants that contain this substance against physical, environmental and microbial harm. The purpose of the present study is to assess the effectiveness of VerbasNol™ in the protection of human skin cells against UV-induced photo damages. The effect of VerbasNol™ on cell proliferation and the expression level of collagen 1 and MMP 1 were analyzed in UV-irradiated human dermal fibroblastic (HDF) cells.

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Materials and Methods

Chemicals

VerbasNol™ powder was dissolved in dimethyl sulfoxide (DMSO). Antibodies against human collagen 1 and MMP1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

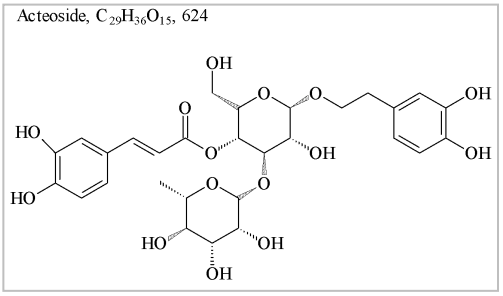

Figure 1. The chemical structure of verbacoside.

Cell culture and cell viability assays

The primary HDF were purchased from Cascade Biologics (Portland, OR, U.S.A.) and cultured in medium 106 supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL of streptomycin. The cells at passages 4-10 were used for this study. The cells were seeded for 24 h before treatment with VerbasNol™, which contains the indicated equivalent verbascoside concentrations, for the indicated time periods. Control cultures were maintained in media supplemented with vehicle (dimethyl sulfoxide (DMSO), 0.1%). No growth or differentiation effects of DMSO were observed under these culture conditions. Cell viability was assessed with CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) following the procedures as described by the manufacturer. Relative cell viability was obtained by measuring absorbances at 450 nm.

UV Irradiation and protection analysis

For UV irradiation, the cells were irradiated with 5 mJ/cm2 of UV-A (315-400 nm) or 20 J/cm2 of UVB (280-315 nm) for HDF cells, using a UV light irradiator (UVItec Unlimited, Cambridge, England) through a colorless and very thin layer of culture medium. The cells were then washed and incubated with serum-free media for 24 h before they were harvested for analysis.

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Western Blot Analysis

To characterize the effect of VerbasNol™ on collagen1 and MMP-1 expression levels, HDF cells were first treated with or without the VerbasNol™ preparations, with verbascoside concentration equivalent to 0.1, 1, and 10 μM, for the indicated time periods. The cells were then washed with ice-cold PBS and lysed in 0.2 mL of lysis buffer (1% NP-40, 150 mM NaCl, 0.1% SDS, 50 mM Tris–HCl, pH 7.6, 10 mM EDTA, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4, 10 mM NaF, 10 mM β-glycerophosphate, 10 μg/mL protease inhibitor, and phosphatase inhibitor cocktails) for 30 min at 4°C. Equal amounts of protein from each sample

were separated by SDS–10% polyacrylamide gel electrophoresis (PAGE) and transblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, U.S.A.). Immunoblotting was performed using antibodies against collagen1 and MMP-1, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Signals were visualized with an enhanced chemiluminescence kit (ECL, Amersham, U.K.) followed by exposure to x-ray films. The protein concentrations of the samples were measured using a bicinchoninic acid (BCA) protein assay kit according to the manufacturer's protocol (Pierce, Rockford, IL, U.S.A.).

Statistical Analysis

Student's t-test for the significance of the difference between the mean values of two independent samples was used. All p values were considered statistically significant at $p<0.05$. Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) 11.0 for Windows software.

Results

1. **The effect of VerbasNol™ on HDF cell proliferation.**

To study the effect of VerbasNol™ on cell proliferation, HDF cells were incubated in the presence or absence of VerbasNol™, under normal control condition (without UV irradiation, Figure 1) or irradiated with UVA (Figure 2) or UVB (Figure 3).

(1) Effect of VerbasNol™ on the proliferation of normal cells

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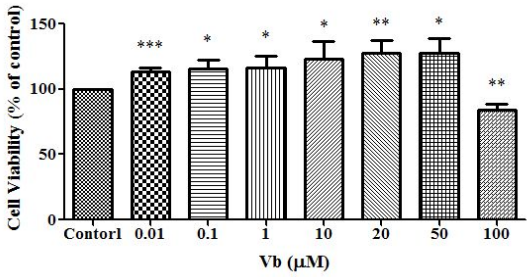

Figure 1. Effect of VerbasNol™ on the proliferation of normal cells

The HDF cells were incubated in the presence or absence of VerbasNol™ with equivalent verbascoside (Vb) concentration as indicated for 24 h. The cells were then washed with

HBSS to remove Vb and left to grow for another 48 h. Cell proliferation was assessed using the CCK-8 reagent kit. Results shown represent mean ± SD (n = 3), with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$, versus control.

(2) Effect of VerbasNol™ on the proliferation of UVA-irradiated cells

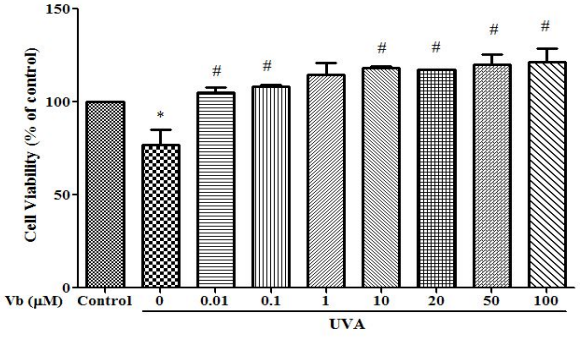

Figure 2. Effect of VerbasNol™ on the proliferation of UVA-irradiated cells

The HDF cells were incubated in the presence or absence of VerbasNol™ with equivalent verbascoside (Vb) concentration as indicated for 24 h. The cells were then washed with HBSS to remove Vb and irradiated with UVA (10 J/cm^2) and left to grow for another 48 h. Cell proliferation was assessed using the CCK-8 reagent kit. Results shown represent mean ± SD (n = 3), with * $p < 0.05$ versus un-irradiated control and # $p < 0.05$ versus UVA-irradiated control.

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(3) Effect of VerbasNol™ on the proliferation of UVB-irradiated cells

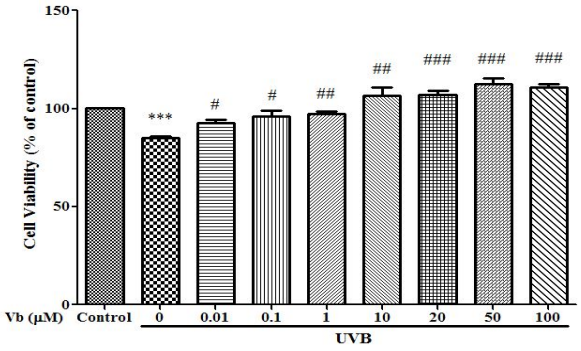

Figure 3. Effect of VerbasNol™ on the proliferation of UVB-irradiated cells

The HDF cells were incubated in the presence or absence of VerbasNol™ with equivalent verbascoside (Vb) concentration as indicated for 24 . The cells were then washed with HBSS to remove Vb and irradiated with UVB (20 mJ/cm^2) and left to grow for another 48 h. Cell proliferation was assessed using the CCK-8 reagent kit. Results shown represent mean ± SD (n = 3), with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ versus un-irradiated control and # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$ versus UVB-irradiated control.

Table 1. Effect of VerbasNol™ on human HDF cells proliferation.

Normal and UVA and UVB irradiated HDF cells were incubated with VerbasNol™ as described in the respective figure legends. Results are summarized from Figure 1 to Figure 3.

VerbasNol™	Cell Proliferation		
0	100%	100%	100%
	Control	UVA	UVB
0	-	76.91%	84.9%
0.01	113.6%***	105.1%#	92.5%#
0.1	115.4%*	108.2%#	96.2%#
1	116.4%*	114.6%	97.2%##
10	123.4%*	118.2%#	106.4%##

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20	127.7%**	117.2%#	107.1%###
50	127.9%*	120.2%#	112.6%###
100	83.0%**	121.5%#	111.0%###

The study on non-irradiated HDF cells incubated with VerbasNol™ exhibits a mild effect on cell proliferation while the studies on UVA and UVB irradiated HDF cells incubated with VerbasNol™ also shown to have similar although weaker cell proliferation effect. No apparent cytotoxicity of VerbasNol™ was shown in these cells, as the cells damaging effect can only be found in cells treated with 100 ug/mL of verbasNol™.

2. **Effect of VerbasNol™ on Collagen 1 and MMP1 expression in HDF cells**.

For this study, both intracellular levels of collagen1 and MMP1 (Figures 4, 6, and 8) and the levels of secreted extracellular forms of these proteins (Figures 5, 7, and 9) were analyzed under the following conditions:

(1) The effect on non-irradiated (control) HDF cells

In this study, the HDF cells were analyzed for collagen 1 and MMP 1 expression, in the presence or absence of VerbasNol™ without UV irradiation. (Figures 4 and 5)

Table 2. Effect of VerbasNol™ on collagen 1 and MMP 1 expression in human HDF cells

Vb (No UV)	Intracellular level		Secreted level	
VerbasNol™	Pro-collagen I	MMP-1	Collagen I	MMP-1
0	100%	100%	100%	100%
0.01	123.7%*	84.89%	154.5%*	100.1%
0.1	145.6%	67.2%	149.8%*	84.%
1	183%*	69.%*	144.9%	79.4%

The results indicated that VerbasNol™ effectively increased collagen I level and inhibited MMP 1

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expression levels. These results suggest that VerbasNol™ displays a protective effect on human skin cells against collagen degradation and MMP-1 expression.

(1-1) Effect of VerbasNol™ on intracellular level of Collagen 1 and MMP 1 in non-irradiated (control) cells

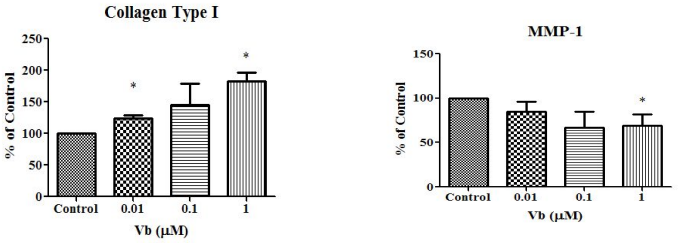

Figure 4. Effect of VerbasNol™ on intracellular Collagen 1 and MMP 1 expression in non-irradiated (control) cells

Human HDF cells were treated with VerbasNol™, with equivalent verbascoside (Vb) concentration as indicated, for 72 h before harvested for analysis. Results shown represent mean ± SD (n = 3), with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ versus un-irradiated control and # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$ versus UVB-irradiated control.

(1-2) Effect of VerbasNol™ on secreted extracellular Collagen 1 and MMP 1 levels on non-irradiated (control) cells

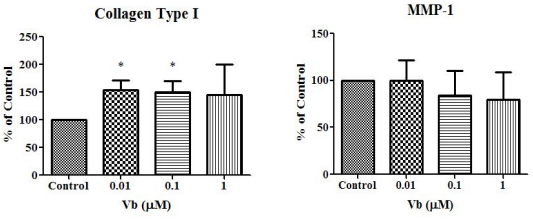

Figure 5 Effect of VerbasNol™ on Collagen 1 and MMP 1 expression on non-irradiated (control) cells

Human HDF cells were treated with VerbasNol™, with equivalent verbascoside (Vb) concentration as indicated, for 72 h before harvested for analysis. Results shown represent mean ± SD (n = 3), with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ versus un-irradiated control and # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$ versus UVB-irradiated control.

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(2) Effect of VerbasNol™ on HDF cells irradiated with UVA

In this study, the HDF cells were irradiated with UVA, in the presence or absence of VerbasNol™ and analyzed for their effect on collagen 1 and MMP 1 expression (Figures 6 and 7).

Table 3. Effect of VerbasNol™ on collagen 1 and MMP 1 expression on UVA-irradiated human HDF cells.

Vb + UVA		Intracellular level		Secreted level	
VerbasNol™		Pro-collagen I	MMP-1	Collagen	MMP-1
0		100%	100%	100%	100%
UVA	0	90.23%	188.1%	77.3%	167.8%
	0.01	98.06%	163.9%	77.61%	145.3%
	0.1	206.2%##	181.8%	99.9%	158.1%
	1	244.4%##	108.1%	90.9%	150.4%

(2-1) Effect of VerbasNol™ on intracellular Collagen 1 and MMP 1 expression on UVA-irradiated cells

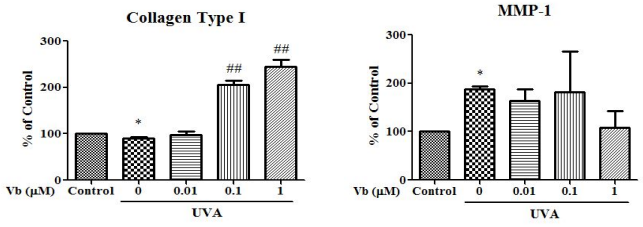

Figure 6. The effect of VerbasNol™ on Collagen 1 and MMP 1 expression on non-irradiated (control) cells

Human HDF cells were treated with VerbasNol™, with equivalent verbascoside (Vb) concentration as indicated, for 72 h before harvested for analysis. Results shown represent mean ± SD (n = 3), with * $p < 0.05$ versus un-irradiated control; # $p < 0.05$ and ## $p < 0.01$ versus UVA-irradiated control.

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(2-2) Effect of VerbasNol™ on secreted extracellular Collagen and MMP levels on UVA-irradiated cells

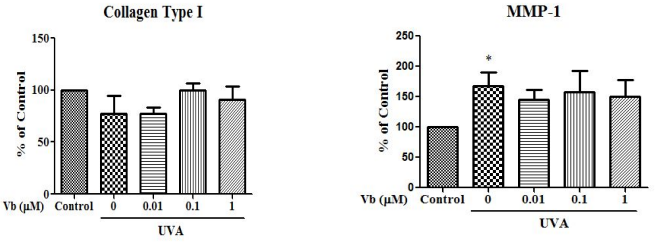

Figure 7. The effect of VerbasNol™ on Collagen 1 and MMP 1 expression on non-irradiated (control) cells

Human HDF cells were treated with VerbasNol™, with equivalent verbascoside (Vb) concentration as indicated, for 72 h before harvested for analysis. Results shown represent mean ± SD (n = 3), with * $p <$ 0.05 versus un-irradiated control.

(3) Effect of VerbasNol™ on UVB-irradiated cells

In this study, the HDF cells were irradiated with UVB, in the presence or absence of VerbasNol™ and analyzed for their effect on collagen1 and MMP1 expression (Figures 8 and 9).

Table 4. Effect of VerbasNol™ on collagen 1 and MMP 1 expression on UVB-irradiated human HDF cells.

Vb + UVB		**Intracellular level**		**Secreted level**	
VerbasNol™		Pro-collagen I	MMP-1	Collagen I	MMP-1
0		100%	100%	100%	100%
UVB	0	61.65%	360.7%	59.6%	218.4%
	0.01	69.03%	144.7%##	79.9%	150.6%#
	0.1	74.86%	156.2%##	82.4%	137.4%#
	1	53.69%	210%	71.4%	192.4%

(3-1) Effect of VerbasNol™ on intracellular Collagen 1 and MMP 1 expression on UVB-irradiated cells

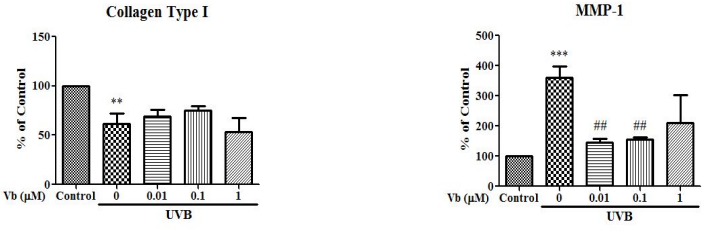

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Figure 8. The effect of VerbasNol™ on Collagen 1 and MMP 1 expression on normal control cells

Human HDF cells were treated with VerbasNol™, with equivalent verbascoside (Vb) concentration as indicated, for 24 h. The cells were washed with HBSS and irradiated with UVB (20 mJ/cm^2) and further incubated for another 48 h. The cells were harvested and the cell extracts were used for analysis.. Results shown represent mean ± SD (n = 3), with * p < 0.05, ** p < 0.01, and *** p < 0.005 versus un-irradiated control; # p < 0.05 and ## p < 0.01 versus UVB-irradiated control.

(3-2) Effect of VerbasNol™ on secreted extracellular Collagen 1 and MMP 1 levels on UVB-irradiated cells

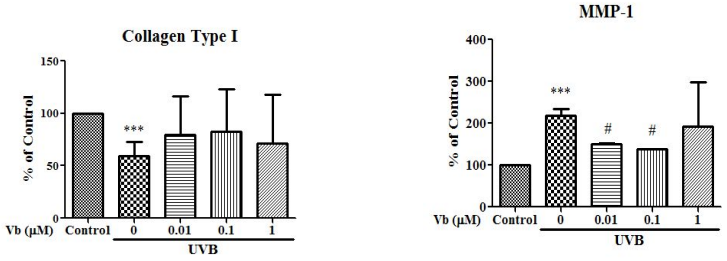

Figure 9. The effect of VerbasNol™ on Collagen and MMP expression on UVB-irradiated cells

Human HDF cells were treated with VerbasNol™, with equivalent verbascoside (Vb) concentration as indicated, for 24 h. The cells were washed with HBSS and irradiated with UVB (20 mJ/cm^2) and further incubated for another 48 h. The culture medium were collected and before harvested for analysis. Results shown represent mean ± SD (n = 3), with * p < 0.05, ** p < 0.01, and *** p < 0.005 versus un-irradiated control; # p < 0.05 versus UVB-irradiated control.

Conclusions

In the total of 9 studies, VerbasNol™ has shown to promote cell proliferation in

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normal and UVA and UVB irradiated HDF cells, to inhibit collagen 1 degradation and MMP 1 expression induced by UV irradiation. The protective effects were observed in both the intracellular and extracellular collagen 1 and MMP 1. These results demonstrated that VerbasNol™ has the potential to protect human skin cells under normal conditions and from photo aging effect under sun exposure.