Article

Food Supplements for Skin Health: In Vitro Efficacy of a Combination of *Rhodiola rosea*, *Tribulus terrestris*, *Moringa oleifera* and *Undaria pinnatifida* on UV-Induced Damage

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Abstract: An increasing number of people seek treatment for aging-related conditions. Plant-derived nutraceuticals are currently of great interest in the setting of dermo-cosmetic studies for their preventive role in photoaging. We conducted an in vitro study on the possible preventive properties against photoaging of a commercially available product (Venerinase®). A mixture of *Rhodiola rosea*, *Tribulus terrestris*, *Moringa oleifera*, *Undaria pinnatifida*, folic acid and vitamin B12 (Venerinase®) was tested for its potential anti-aging effects on the skin in vitro. Conventional histology, immunofluorescence and real time PCR were employed in the research protocol. The tested product was proven to prevent UV-induced morphological changes both in keratinocytes and fibroblasts. Moreover, senescence-related and proinflammatory pathways commonly triggered by UV exposure were demonstrated to be inhibited by Venerinase® pretreatment. Our results support the potential clinical benefits of oral supplements for the treatment and/or prevention of cutaneous photodamage.

Keywords: nutraceuticals; oral supplements; *Rhodiola*; *Tribulus*; *Moringa*; skin aging; photoaging; inflammation; senescence; UV damage

1. Introduction

Skin aging is a complex biological process characterized by progressive skin changes, commonly further classified as intrinsic or extrinsic aging. Intrinsic skin aging, also known as chronological cutaneous aging, is due to the passing of time. Chrono-aging can be influenced by several factors including the skin phototype, ethnicity, telomere length and hormonal and genetic factors [1–3]. With regard to cutaneous pigmentation, for example, not only are non-melanoma skin cancers (NMSCs) far rarer in dark-skinned individuals, but a lower variability in terms of skin quality is also observed between sun-exposed and non-exposed areas. Taken together, these data emphasize the close relationship between intrinsic and extrinsic factors in cutaneous aging [1,2]. On the other hand, external factors play a pivotal role in extrinsic aging, with chronic sun exposure and ultraviolet (UV) irradiation determining progressive damage to the skin [4]. While UVB rays cause inflammatory changes and DNA damage in the epidermis and papillary dermis, UVA rays induce dermal elastosis through an oxidative-stress-mediated mechanism [5]. However, UVB and UVA rays often share common biological effects and frequent overlaps in terms of the clinical consequences

of chronic exposure. Clinical manifestations of cutaneous aging include seborrheic keratoses, solar lentigo, wrinkles, yellowing of the skin, Favre–Racouchot disease, actinic keratoses and skin cancers [6,7].

From a more microscopic point of view, cutaneous aging is characterized by epidermal and dermal thinning, reduced cutaneous vascularization, loss and/or fragmentation of collagen, elastic fibres and proteoglycans [8].

Due to the recent increase in lifespan, not only do more and more patients seek treatment for cutaneous malignancies, but they also return to aesthetic procedures, cosmetic products and oral supplements to prevent and/or treat aging-related dermatological changes [9,10].

Oxidative stress has been demonstrated to play a key role in both skin photo- and chrono-aging; therefore, antioxidants are nowadays considered valid therapeutic tools in the field of skincare [11,12]. Ascorbic acid (commonly referred to as vitamin C) is probably the most renowned antioxidant worldwide and exerts a protective role on the skin from sun damage [13,14]. Vitamin C is present both in oral and topical formulations used for antiaging purposes.

An increasing body of evidence points at nutraceuticals for the prevention of aging-associated skin changes [15–17] and natural extracts are currently being investigated for their potential inhibiting action on aging [18,19]. Several natural leaves—including moringa, curry, guava, ginko biloba, olive, grape, green tea, *Tribulus terrestris*, wakame and roseroot leaves—have been widely studied for their benefits in the setting of aging science [20,21]. Their protective role against aging and age-related comorbidities is due to a large variety of bioactive components, the most well-known being quercetin, catechins, flavonoids, polyphenols, anthocyanins, terpenoids, tannins, glutathione, melatonin and other glycosides [12,21]. Various types of in vitro and animal models have already been employed so far with the aim of exploring the possible mechanisms of action of these substances [22,23]. However, current evidence is often limited to the study of a single anti-aging molecule and/or natural extract.

The reduced bioavailability of vitamin B12 and folic acid metabolism impairment have also been demonstrated in aging [24–26] and supplementation with folates and B12 has been demonstrated to possess anti-aging effects in experimental models [27–29]. Other proposed treatments for treating aging-associated manifestations include metformin, stem cells and hyaluronic acid (either injected or topically applied), as well as other cosmetic and aesthetic procedures [30–33].

The aim of the present study was to assess the in vitro effects of a commercially available over-the-counter dietary supplement containing a combination of four natural leaf extracts, vitamin B12 and folic acid on skin aging (Venerinase®; Cristalfarma srl, Milan, Italy). The ingredients of Venerinase® on their own have already been proposed as possible preventive tools against photoaging and the recent literature points at *Moringa oleifera*, *Rhodiola rosea*, *Undaria pinnatifida* and *Tribulus terrestris* as useful therapeutic tools based on in vitro evidence of their antiaging action [22,34–37]. However, similar scientific data are currently lacking for combinations of these products.

2. Materials and Methods

2.1. Experimental Device

A mixture of *Rhodiola rosea*, *Tribulus terrestris*, *Moringa oleifera*, *Undaria pinnatifida*, folic acid and vitamin B12 as an analogue to the commercially available Venerinase® (Cristalfarma srl, Milan, Italy; for final composition see Table 1) was prepared and tested for its potential anti-aging effects in vitro.

Table 1. Venerinase[®] composition corresponding to the reference daily intake. Each component was dissolved in its solvent as indicated.

Components	mg	Solvent
Rhodiola rosea	400	EtOH 50%
Tribulus terrestris	350	H_2O
Moringa oleifera	150	H_2O
Undaria pinnatifida	50	H_2O
Folic acid	0.2	NaOH 0.1 N
Vitamin B12	0.0025	EtOH 50%

2.2. Fibroblast Culture and UV Irradiation Treatment with Venerinase[®]

Human fibroblasts (C0135C, Thermo Fisher Scientific, Waltham, MA, USA) were expanded up to passage 3, then culture medium was added with Venerinase[®] at different concentrations for 24 h to subsequently determine a suitable working dose without affecting cell viability and proliferation. To this purpose, human fibroblasts underwent a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric test [38]. With regard to this, cells were incubated for 3 h with MTT reagent at 37 °C. After incubation, the purple formazan crystals were dissolved in DMSO at room temperature, then the absorbance was measured at OD = 590 nm by using a multi-well plate reader (Thermo Scientific Appliskan, Thermo Fisher Scientific). The determined working concentration was applied for 24 h to fibroblast cultures, then cells underwent UV irradiation at a dose of UVB of 50 mJ/cm^2 (Philips—TL 20W/12RS, 270–420 nm) [39,40].

2.3. Evaluation of Fibroblast Aging and Photodamage

In order to evaluate the effects of UV exposure on human fibroblasts, real time PCR (rt-PCR) analyses were performed to assess the mRNA levels of ACTA2, HDAC4, SPARC, p21, IL-6 and TNF-α, as previously described [41]. Cells were homogenized and the total RNA was extracted and purified using PureLink RNA columns (Thermo Fisher Scientific). The RNA integrity and quantification were analyzed via a spectrophotometric method by using a NanoDrop 2000 device (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 µg) was reverse transcribed to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The levels of mRNA were quantitatively determined (for PCR primer sequences see Table 2) on a QuantStudio 3 Real Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) using a QuantiFast SYBR Green PCR Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Furthermore, immunofluorescence (IF) staining for specific photodamage markers, such as p27, α-SMA and COX-1, was also performed. Cells were fixed with 4% para-formaldehyde at 4 °C for 15 min and then permeabilized with 0.3% Triton X-100 for 5 min. After blocking with 3% bovine serum albumin (BSA) in pH 7.4 phosphate-buffered saline (PBS) for 1 h, the cells were incubated at 4 °C overnight with the primary antibodies: rabbit anti-p27 (1:50; Abcam), mouse anti-α-SMA (1:100; Invitrogen, Waltham, MA, USA) and rabbit anti-COX-1 (1:50; StressMarq Biosciences, Victoria, BC, Canada). Then, after washing in BSA 1% in PBS, cells were subsequently incubated for 1 h at room temperature with the following secondary antibodies: goat anti-rabbit Alexa488 and goat anti-mouse Alexa546 (all diluted 1:200; Thermo Fisher Scientific). Nuclei were counterstained with 1 µM 4,6-diamidino- 2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). In parallel, the cell morphology was evaluated by immunolabeling with TRITC-conjugated anti-phalloidin antibody (Abcam, Cambridge, UK). Samples were observed with a Nikon A1 confocal laser scanning microscope (Nikon, Minato, Tokyo, Japan) and image rendering was performed using ImageJ 2.9.0 and Adobe Photoshop Software 7.0 [42].

Table 2. List of PCR primers sequences used.

Target Gene	Forward Sequence	Reverse Sequence
ACTA2	AATGCAGAAGGAGATCACGG	TCCTGTTTGCTGATCCACATC
HDAC4	ACAAGGAGAAGGGCAAAGAG	GCGTTTTCCCGTACCAGTAG
SPARC	CAAGAAGCCCTGCCTGATGA	TGGGAGAGGTACCCGTCAAT
p21	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG
IL-6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
TNF-α	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC

2.4. Epidermal Aging Assay

Commercially available epidermis (EpiDerm™, Mattek, Ashland, MA, USA) was pre-treated with single (at t0) and double (at t0 and after 24 h, t24) doses of Venerinase® before UVB exposure, according to the aforementioned protocol. After 24 h, UVB-irradiated EpiDerm™ samples were processed for paraffin embedding. Briefly, samples were fixed in 4% paraformaldehyde in PBS, washed with PBS and dehydrated with graded ethanol, cleared and embedded in paraffin. Five-micrometre-thick serial cross-sections of the specimens from each experimental group were obtained. Routine haematoxylin/eosin staining was performed in order to analyse the morphological details of the EpiDerm samples from each group, i.e., epidermal stratification and differentiation.

Histological images were obtained using a Nikon Labophot-2 light microscope with a DS-5Mc CCD camera.

IF staining was performed by labelling epidermis sections with mouse anti-PCNA (Millipore, Burlington, MA, USA), rabbit anti-CK-1 (Abcam) and rabbit anti-Beclin-1 (Stress-Marq Biosciences) to assess proliferation, cytoskeleton integrity and cell damage, as described above. In order to evaluate the nuclear damage, the total RNA content was also measured for DNA transcription assessment. Finally, real-time PCR analyses of IL-6 and TNF-α expressions were repeated on epidermis samples after UV exposure to investigate the potential anti-inflammatory effects of Venerinase® (for PCR primer sequences see Table 2).

2.5. Statistical Analysis

Experiments were performed in triplicate and the results were expressed as means $\pm$ SD. A student's *t* test was carried out to evaluate differences between two groups. Conversely, differences among three or more groups were analysed through an ANOVA, followed by a Newman–Keuls post hoc test. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Cell Viability

The MTT test showed good tolerability of the Venerinase® product, with only a slight (non-statistically significant) reduction in cell viability at high dose concentrations (1:50/2%). After evaluating different concentrations, 0.1% Venerinase® was proven to be the most suitable for the subsequent assays (Figure 1).

3.2. Venerinase®-Induced Changes in the Dermal Compartment

As shown in Figure 2A, UV exposure induced a shift in cell morphology of cultured fibroblasts, which became more dendritic and/or flattened (myofibroblast shape). Notably, 0.1% Venerinase® was able to revert UV-induced morphological changes and to restore the normal spindle shape fibroblasts. Pre-treatment of fibroblasts with the Venerinase® reference daily intake (see Table 1) also determined a statistically significant reduction in the expression of early fibrotic markers such as HDAC4 and SPARC when compared to the UV-treated group (° *p* < 0.05 vs. UV), as demonstrated by real-time PCR analyses, whereas

no statistically significant changes were observed in the mRNA expression levels of ACTA2 (Figure 2B).
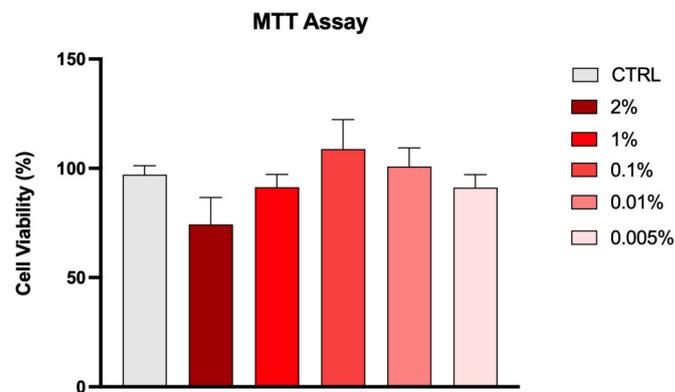
Figure 1. Evaluation of cell viability in fibroblasts. Cell viability (expressed as % of viable cells) in fibroblasts treated with different concentrations of Venerinase® (2%, 1%, 0.1%, 0.01%, 0.005%) was investigated to define the working concentration to be used for the subsequent experimental evaluations. Data are expressed as means ± standard deviation (SD) and analysed by a one-way analysis of variance (ANOVA) followed by a Newman–Keuls post hoc test. Statistically significant differences were set at $p < 0.05$.
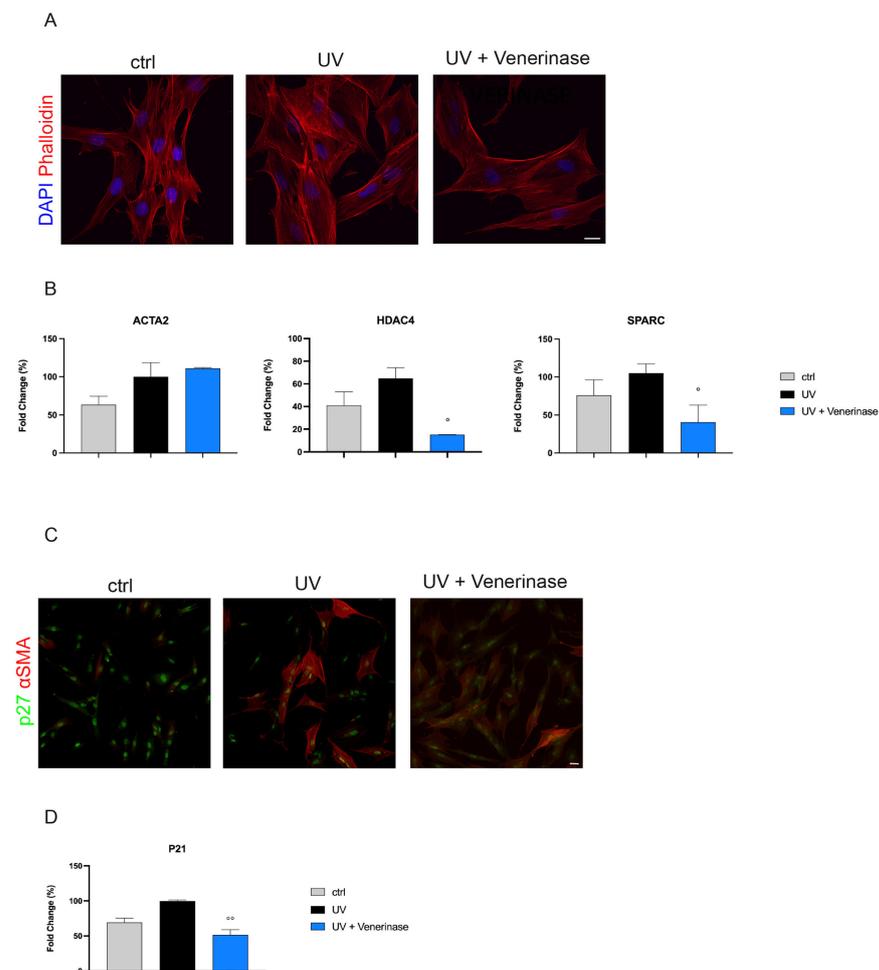

Figure 2. Evaluation of Venerinase® pre-treatment effects on UV-exposed human fibroblasts. (**A**) A cell morphology analysis was carried out through phalloidin staining. Nuclei were counterstained with

DAPI (blue). Scale bar: 10 μm. (**B**) Real time PCR analyses of fibrosis-associated markers ACTA2, HDAC4 and SPARC. Histograms report the percentage fold change in the mean values of mRNA levels ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. ° $p < 0.05$ vs. UV. (**C**) Confocal immunofluorescence analysis for p27 and α-SMA. Scale bar: 10 μm. (**D**) Real time PCR analysis of mRNA levels of p21. Histograms represent mean values ± standard deviation (SD). One-way ANOVA followed by Newman–Keuls post hoc test. °° $p < 0.01$ vs. UV.

Moreover, Venerinase®-pretreated fibroblasts displayed a reduced α-SMA expression, with a parallel increase in P27 compared to non-treated UV-exposed cells, therefore suggesting Venerinase® possibly prevents the acquisition of a pro-fibrotic phenotype (Figure 2C).

At the same time, the expression of the senescence marker p21 assessed by a real-time PCR analysis was reduced by Venerinase® pretreatment (°° $p < 0.01$ vs. UV; Figure 2D).

With regard to inflammatory markers, real time PCR data confirmed statistically significant fold-change reductions in the mRNA levels of TNF-α and IL-6 (Figure 3A). In parallel, IF also showed a reduced expression of COX-1, thus confirming the possible anti-inflammatory role of Venerinase® (Figure 3B).
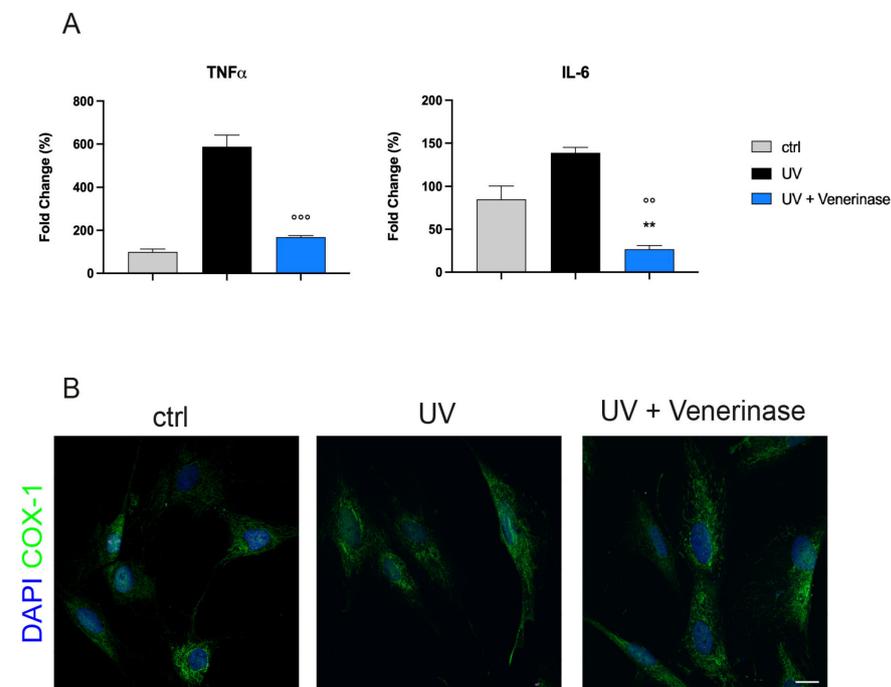
Figure 3. Inflammatory cytokines and oxidative stress in human fibroblasts after Venerinase® pretreatment and UV exposure. (**A**) Real-time PCR analyses of inflammatory cytokines TNFα and IL-6 in human fibroblasts. Histograms report the percentage fold change in mean values of mRNA levels ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. °° $p < 0.01$, °°° $p < 0.001$ vs. UV; ** $p < 0.01$ vs. ctrl. (**B**) Confocal immunofluorescence analysis of COX-1. Nuclei were counterstained with DAPI (blue). Scale bar: 10 μm.

3.3. Venerinase®-Induced Changes in the Epidermis

The haematoxylin and eosin staining in Figure 4A shows that UV exposure induced cornification of the epidermis, with a significant reduction in the number of nuclei in the lower epidermal layers 24 h after UV irradiation. Normal stratification of the epidermis was restored by pre-treatment with Venerinase® at either a single or double dose (Figure 4A).
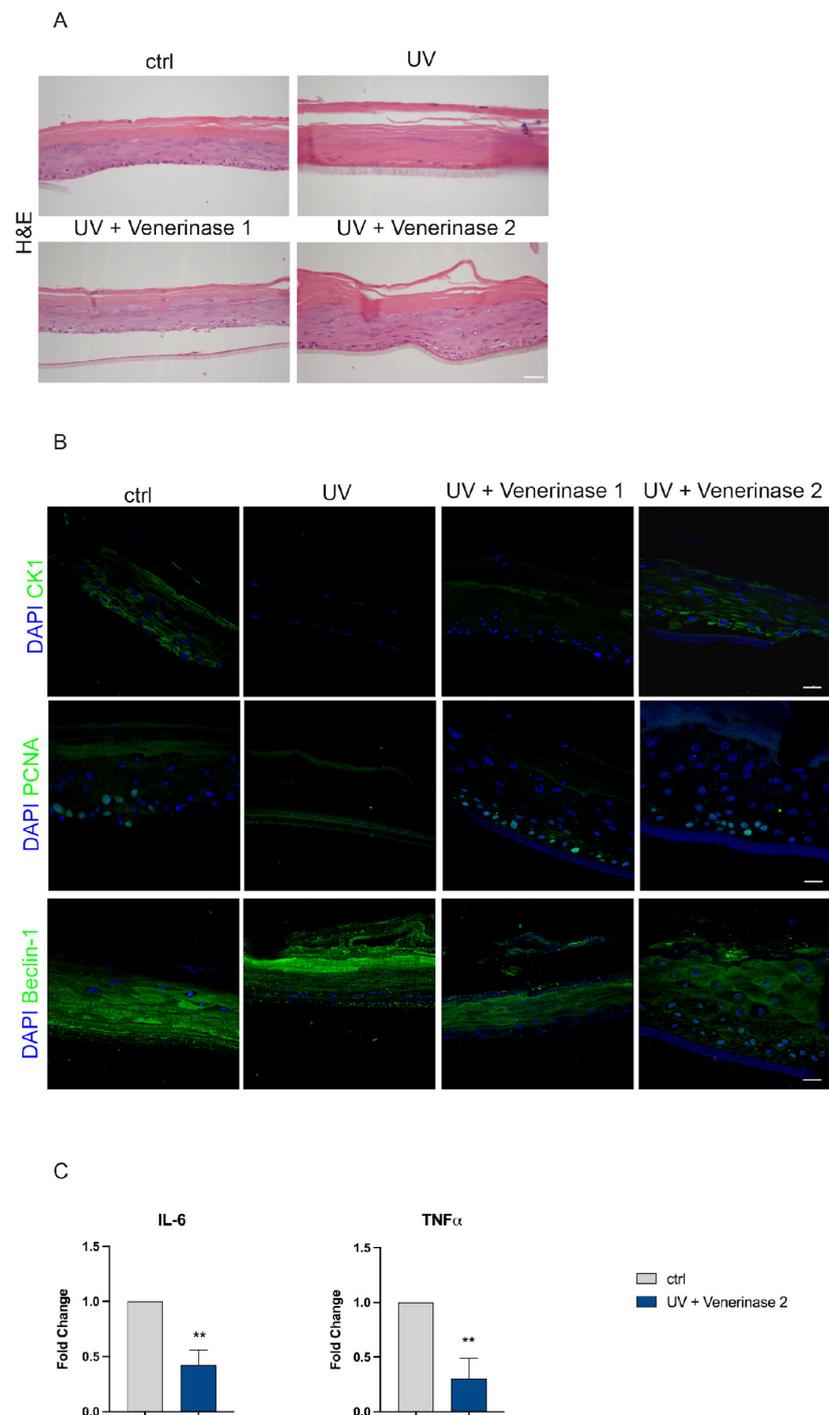
Figure 4. Protective effects of Venerinase® pre-treatment on UV-exposed epidermis. (**A**) Histological analysis of EpiDerm samples from each experimental group. H&E staining showing the effects of Venerinase® pre-treatments (Venerinase® 1, corresponding to t0 administration and Venerinase® 2 corresponding to t0 and t24 h administration) on UV-irradiated EpiDerm stratification. Scale bar: 50 μm. (**B**) Confocal analysis of CK1, PCNA and beclin-1. Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm. (**C**) Real-time PCR analyses of IL-6 and TNFα in UV-exposed EpiDerm previously treated with a double dose of Venerinase®. Histograms report the fold change in mRNA levels indicated as means ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. ** $p < 0.01$ vs. ctrl.

The IF analyses in Figure 4B show that CK1, normally expressed in epidermal suprabasal layers, is lost secondary to UV exposure, indicating a loss of normal cell morphology. Interestingly, a single dose of Venerinase® promoted an early reversion of UV-induced changes, which was more appreciable when a double dose was administered. PCNA, expressed by basal keratinocytes in control samples, was absent in UV-damaged skin. Epidermal exposure to Venerinase® before irradiation caused a significant enhancement in the PCNA expression in the basal layer both after single and double dose treatments (Figure 4B). Beclin-1, normally detected in the cytosol of healthy keratinocytes, reduced its expression following UVB exposure. Following Venerinase® pre-treatment with a double dose, beclin-1 nuclear translocation was observed in the epidermis.

As for DNA transcription, the total RNA concentration was higher in all Venerinase®-treated samples compared to non-treated UV-exposed skin (Table 3), suggesting the protective role of Venerinase® pre-treatment in terms of photodamage prevention at the nuclear level. Such evidence was also accompanied by statistically significant reductions in proinflammatory cytokine levels, including both IL6 and TNF-α (** $p < 0.01$ vs. ctrl; Figure 4C).

Table 3. Extraction and quantification of RNA from EpiDerm samples. CTRL: untreated EpiDerm; UV: UV-exposed EpiDerm; UV + Venerinase® 1: UV-exposed EpiDerm pre-treated with a single dose of Venerinase®; UV + Venerinase® 2: UV-exposed EpiDerm pre-treated with a double dose of Venerinase®.

	Tissue Weight (mg)	RNA Concentration (ng/µL)
CTRL	3	136
UV	3.1	2.4
UV + Venerinase® 1	4.5	39.1
UV + Venerinase® 2	4.7	133.2

4. Discussion

The present study clearly demonstrates the beneficial effect in vitro on skin photoaging of pretreatment with a mixture of *Rhodiola rosea*, *Tribulus terrestris*, *Moringa oleifera*, *Undaria pinnatifida*, folic acid and vitamin B12.

Notably, UV exposure determines direct cell injury to keratinocytes and fibroblasts, mostly mediated by DNA damage. In line with this, our preliminary results on cell viability showed that UVB doses higher than 50 mJ/cm^2 on cell monolayers determine cell damage and apoptosis after 24 h from exposure.

We then took into consideration the results collected after preventive treatment with Venerinase®. Interestingly, Venerinase® was able to reduce the pathological expressions of pro-fibrotic markers in fibroblasts induced by UV exposure, such as HDAC4 and SPARC, which are commonly produced upon pro-inflammatory stimuli and are therefore possibly associated with the so-called "inflammaging" [43,44]. The cytoprotective effects of Venerinase® were also confirmed by reduced myofibroblast phenotype acquisition by dermal fibroblasts, as shown by the decreased expression and cytoskeletal arrangement of α-SMA. Interestingly, Venerinase® pretreatment before UV exposure also reduced the expression of P21 in fibroblasts. It is well known that P21 is a powerful inhibitor of cyclin-dependent kinases, and is therefore considered a marker for cell senescence [45]. P21 also interacts with PCNA, a processivity factor for DNA polymerase with a regulatory function in DNA repair and replication during cell cycle; its enhanced expression is therefore possibly associated with DNA damage [46]. Even more importantly, pretreatment with Venerinase® determined significant reductions in the production of inflammaging-associated cytokines, such as TNFα and IL-6, compared to the non-treated controls after UV exposure both in the dermal and epidermal compartments [47]. A reduced COX-1 expression in the treatment group confirmed these results and indicated a reduced oxidative stress induction, thus highlighting the significant antioxidant action of this product [48]. In normal conditions,

COX-1 displays mitochondrial expression, whereas a signal cytosolic translocation was observed after UV exposure [49]. Such a phenomenon was at least partially prevented by the use of Venerinase®.

When we looked more into detail at UV-induced changes in the epidermis, we noticed altered stratification and cell morphology, with cytoskeleton rearrangement possibly being a major contributing factor. A key role in the epidermal UV response is also played by beclin-1. Beclin-1 is a key activator in the initiation of autophagy and also seems to be involved in the regulation of skin pigmentation through melanosome degradation in both keratinocytes and melanocytes [50,51]. Not surprisingly, changes in the beclin-1 expression are observed after UV exposure.

All the aforementioned changes in the epidermal compartment are at least partially restored by pretreatment of keratinocyte sheets with Venerinase®.

Some evidence already exists on the efficacy of the single ingredients of the aforementioned formulation as antiaging therapeutic tools, the most studied certainly being *Moringa oleifera*.

A metabolomic analysis of *Moringa oleifera* leaves identified eight main groups of key metabolites, possibly explaining the moringa-mediated antiaging and anti-inflammatory action [52]. These include carotenoids, kaempferol, quercetin, glucosinolates, sulfolipids, fatty acyl amides and apigenin-O/C-glycosides.

Another recent publication revealed that *M. oleifera* leaf extracts possess good activities against skin-aging-related enzymes [53]. Elastase and collagenase inhibition was demonstrated through spectrophotometric and fluorometric assays, followed by ultrafiltration coupled to multi-target bio-affinity ultrafiltration and high-performance liquid chromatography–mass spectrometry for the identification of possible specific bioactive components with anti-aging properties.

Several authors have also tried to enhance *Moringa*'s bioavailability in order to boost its efficacy. With regard to this, flexible nanoliposome entrapment of isothiocyanates from the moringa tree demonstrated enhanced skin permeation and significant reductions in the expression of UVB-induced reactive oxygen species and matrix metalloproteinases [54].

The anti-aging effects of *Moringa oleifera* seem not to be limited to the skin. In a rat model, oral administration of an aqueous extract of *M. oleifera* leaves (200 mg/kg) for 30 days determined significant reductions in lipid peroxidation and lipofuscin pigmentation along with elevated serotonin and antioxidant enzymes in the brain tissue [55]. Other studies confirmed that *Moringa* alleviates stress and has a neuroprotective function in murine models [56]. These results suggest a beneficial role of oral supplementation with potential positive consequences far beyond the skin.

As for the other components of the Venerinase® product, the literature on natural remedies for preventing cutaneous aging also presents some evidence for this effect in *Rhodiola* and *Undaria*, while little is known with regard to *Tribulus terrestris'* effects on the skin [22,36,37].

Plant extracts containing *Rhodiola rosea* have been demonstrated to extend lifespan in lower model organisms through IGF (insulin growth factor) signalling and have an antioxidant action [57–59]. *Rhodiola*-mediated antagonistic action on oxidative stress induced by UV has also been confirmed by in vitro studies on fibroblasts [35,36,60]. Last but not least, *Undaria pinnatifida* is a source of fucoidan, which has been shown to exert protective action against UV-induced photodamage in vitro due to inhibition of mitochondrial dysfunction mediated by the SIRT-1/PGC-1α signalling pathway [22,61].

5. Conclusions

The combination of *Rhodiola rosea, Tribulus terrestris, Moringa oleifera, Undaria pinnatifida*, vitamin B12 and folic acid was effective in the prevention of UV-mediated damage in vitro, thus supporting the potential clinical benefits of oral supplementation. Such extracts gave excellent results in vitro on cutaneous aging in terms of UV damage prevention both in the dermal and epidermal compartments. Further studies are needed to weigh the contribution

of the single components in the global anti-aging effect and to possibly optimize and improve the efficacy of plant-based dietary supplements in this field.

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