Protective effects of an extract of the freshwater microalga
Scenedesmus rubescens on UV-irradiated skin cells

R. Campiche*, P. Sandau†, E. Kurth†, M. Massironi‡, D. Imfeld§ and R. Schuetz*
†DSM Nutritional Products, Personal Care & Aroma, Wurmisweg 576, CH-4303 Kaiseraugst, Switzerland, §IGV Institut für Getreideverarbeitung
GmbH, Arthur Scheunert Allee 40-41, D-14558 Nuthetal, Germany and ‡Citech srl, Via San Marco 9/M, I-35129 Padua, Italy

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Abstract

BACKGROUND: Skin ageing results from intrinsic but also extrinsic factors of which UV irradiation is a main cause. It is hence of interest to have means to protect skin from UV irradiation-induced damage. We selected an extract of the freshwater microalga Scenedesmus rubescens and assessed its potential to protect skin from photoageing caused by UV irradiation.

METHODS: Skin cells in vitro and ex vivo were analysed for markers of UV irradiation-induced photodamage such as decreased viability, decreased collagen content, hyperpigmentation and sunburn cells.

RESULTS: We found that a dry extract of the microalga Scenedesmus rubescens was able to suppress cellular signs of ageing induced by UV irradiation. It enhanced dermal fibroblast vitality, rescued dermal collagen content, inhibited the formation of sunburn cells and inhibited tyrosinase activity.

CONCLUSION: An extract of Scenedesmus rubescens showed broad activity against markers of UV irradiation-induced cutaneous ageing. It may therefore be used as a preventive or regenerative agent for anti-ageing strategies.

Résumé

CONTEXTE: Le vieillissement cutané résulte de facteurs intrinsèques mais aussi extrinsèques et l’exposition aux UV en est une cause principale. Il y a donc tout intérêt à disposer de moyens pour protéger la peau des dommages induits par l’exposition aux UV. Nous avons sélectionné un extrait de la microalgue d’eau douce Scenedesmus rubescens et évalué ses capacités à protéger la peau du photo vieillissement causé par l’exposition aux UV.

MÉTHODES: Des cellules de peau in vitro et ex vivo ont été analysées sur des marqueurs de photo vieillissement induits par l’exposition aux UV tels que la baisse de la viabilité cellulaire, la baisse de la teneur en collagène, l’hyperpigmentation et le nombre de cellules coup de soleil (« sunburn cells »).

RÉSULTATS: Nous avons trouvé qu’un extrait de la micro algue Scenedesmus rubescens était capable de supprimer les signes cellulaires de vieillissement induits par l’exposition aux UV. Il améliore la viabilité des fibroblastes dermiques, préserve la teneur en collagène dans le derme, inhibe la formation de « sunburn cells » et inhibe l’activité de la tyrosinase.

CONCLUSION: Un extrait de Scenedesmus rubescens a montré une large activité sur les marqueurs du vieillissement cutané induit par l’exposition aux UV. Il peut par conséquent être utilisé comme un moyen préventif ou régénérateur dans les solutions anti-âge.

Introduction

Skin ageing can result from endogenous as well as exogenous influences. These include such diverse factors as solar irradiation, air pollution, cigarette smoke, stress, nutrition, lack of sleep and temperature. These factors were recently termed the skin ageing exposome [1]. Despite the many different factors contributing to skin ageing, solar irradiation is still believed to be the number one cause of cutaneous ageing [2]. Within solar irradiation, ultraviolet radiation (UVR) is the main contributor, although recently visible light, in particular high-energy visible (HEV) light, with wavelengths between 400 and 500 nm has also gained attention [3]. UVR is divided into UVC (<290 nm), UVB (290–315 nm) and UVA (315–400 nm). While UVC is absorbed completely in the atmosphere and does not reach earth’s surface, both UVB and UVA together with the rest of the solar spectrum (visible and infrared) reach the earth’s surface. Life without solar radiation would not be possible. With respect to human skin, UVR is essential for the synthesis of vitamin D [4]. Nevertheless, UV irradiation is attributed to many physiologically adverse effects and it is the main reason for the skin’s ageing process.

Typical signs of photoageing evoked by UV irradiation are the formation of wrinkles, a loss of skin firmness and elasticity, as well as uneven skin tone and age spots [5] together with dry skin conditions [6]. Intrinsic changes, representing a generalized skin atrophy with structural changes, occur in all skins as people age. Photoageing, on the contrary, evokes a high amount of molecular and cellular responses leading to solar skin damage. Photoageing hence is the superposition of solar damages on the intrinsic skin ageing process [7, 8]. The underlying mechanisms include a decrease in dermal collagen content by upregulation of matrix metalloproteinases (MMPs) [9, 10], solar elastosis [11], as well as hyperpigmentation [12]. These outcomes can go along with increased inflammation leading to inflammation [13], as well as oxidative stress through the formation of reactive oxygen species (ROS) [14]. There is also DNA damage and cellular apoptosis, for example in the form of sunburn cells [15]. Other, more clinically relevant, outcomes of solar irradiation are the development of actinic keratosis and, more seriously, the

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development of various types of skin cancers such as squamous cell carcinoma or melanoma [16]. Therefore, potential help against photoaging, in addition to chemical UVR filters, may come from molecules with biological activity against oxidative stress, inflammation and DNA damage, as well as hyperpigmentation, for example antioxidants and depigmenting agents, such as vitamins or tyrosinase inhibitors.

Our aim was to investigate the potential of microalgal extracts as protective agents against ageing markers of UV irradiation. Microalgae are a rich and renewable source of molecules with applications in nutrition, medicine and cosmetics, among others. They contain diverse bioactive compounds dedicated to survival in complex and extreme environments, such as heat, drought or exposure to sunlight. The various biological activities reported include antioxidant, anti-inflammatory and anti-microbial activity [17]. The study presented here describes the results obtained with an aqueous extract of the freshwater microalga Scenedesmus rubescens. Extracts of Scenedesmus have been shown to be rich in protein, carbohydrate and lipids [18], and the cells are used in biodiesel production [19]. The findings we present here also show broad activity of the extract of Scenedesmus rubescens against UV irradiation-induced cellular damage.

Materials and methods

Scenedesmus rubescens dry extract preparation

Scenedesmus rubescens biomass was suspended in H2O and heated to 100°C for 2 h. After centrifugation, the liquid phase was ultrafiltrated and dried. Test samples were prepared by reconstitution of dry extract in either an aqueous solution or DMSO.

UV spectrum analysis

The Scenedesmus rubescens dry extract was diluted in 100 µL water at a concentration of 100 µg/mL and plated in a 96-well plate. The spectrum was analysed by measurement of spectrophotometer absorbance with the BioTek Synergy 2 (BioTek, Luzern, Switzerland) in the range of 200–700 nm (each step 10 nm).

Cell viability assay in vitro

Human dermal fibroblasts from abdominal surgery were cultured between passages 8 and 14 and were maintained in Dulbecco’s modified Eagle’s medium high glucose (DMEM) (Gibco Invitrogen, Zug, Switzerland) containing 10% foetal calf serum (FCS) (Amimed, BioConcept, Allschwil, Switzerland) and 1% penicillin/streptomycin (P/S) (Invitrogen, Zug, Switzerland). Cells were cultured at 37°C in a humidified 5% CO2-air atmosphere, incubated with Scenedesmus rubescens dry extract reconstituted in cell culture medium. Total collagen content was measured by picrosirius red staining after 48 h.

Analysis of total collagen content in vitro

Human dermal fibroblasts from abdominal surgery were cultured between passages 8 and 14 and were maintained in Dulbecco’s modified Eagle’s medium high glucose (DMEM) (Gibco Invitrogen, Zug, Switzerland) containing 10% foetal calf serum (FCS) (Amimed, BioConcept, Allschwil, Switzerland) and 1% penicillin/streptomycin (P/S) (Invitrogen, Zug, Switzerland). Cells were cultured at 37°C in a humidified 5% CO2-air atmosphere, incubated with Scenedesmus rubescens dry extract reconstituted in cell culture medium. Total collagen content was measured by picrosirius red staining after 48 h.

Analysis of tyrosinase inhibition in vitro

Tyrosinase activity was measured in a cell-free assay. The measurement principle was the conversion of DOPA to dopachrome by mushroom tyrosinase (Sigma, Buchs, Switzerland). Dopachrome analysis took place at 475 nm in an absorbance plate reader.

For the following ex vivo experiments, skin biopsies from a 43-year-old Caucasian female donor from abdominal plastic surgery were acquired following the guidelines of the Helsinki declaration and after obtaining the patients’ informed consent. The skin phototype of the donor was classified as brown, having an ITA° angle of 9.3 as described in Del Bino et al. [20].

Analysis of collagen III content ex vivo

Twelve tissue samples per treatment measuring 8 x 3 mm were cultured for 6 days. Dry extract of Scenedesmus rubescens solubilized in DMSO (4 µL) was applied topically, covered with a delivery membrane 6 mm in diameter (CoTran delivery membrane, 3M, Pioltello, Italy, cat#9728) and renewed daily. Tissues were formalin fixed and paraffin embedded. Skin sections were immunostained with a mouse monoclonal antibody against collagen III (Sigma, Buchs, Switzerland, #C7805). The secondary antibody we used was a biotinylated secondary antibody of the kit Dako real (TM) detection system, alkaline phosphatase/RED and rabbit/mouse (DAKO, #K5005). The amount of antigen present is evaluated by estimating the intensity and the distribution of the red staining within the dermis semiquantitatively using ImageJ software (NIH-USA). As a reference compound, we used retinoic acid (Sigma, Buchs, Switzerland, #R2625) at 0.05% in DMSO.

Analysis of sunburn cells after UV irradiation ex vivo

Twelve tissue samples per treatment measuring 8 x 3 mm were cultured for 3 days. Dry extract of Scenedesmus rubescens solubilized in DMSO (4 µL) was applied topically, covered with a delivery membrane 6 mm in diameter (CoTran delivery membrane, 3M, Pioltello, Italy, #9728) and renewed daily. As a control, we used a commercially available sunscreen (Eucerin 50+ kids sunscreen lotion claiming UVB and UVA protection).
applied topically (4 μL). At day 2, the application of test products and control was renewed. Immediately before irradiation, excess formulation was gently wiped off, and UVB (1 J/cm²) from a Bio-Sun (Vilber-Lourmat, Eberhardzell, Germany) was applied, and after 24 h, the biopsies were harvested, formalin fixed and paraffin embedded. The UVB fluency was determined according to the biologically effective dose (BED) described by Del Bino et al. [20]. The UVR source emitted primarily in the UVB range (280–315 nm, peak at 312 nm (Fig. 1)) and elongated into UVA range (315–400 nm). Sunburn cells were stained by haematoxylin and eosin staining and counted manually on skin sections.

Statistical analysis
Measurement samples were tested for normal distribution by the Shapiro–Wilk test followed by Student’s t-test for unpaired samples to test for significant differences between samples.

Results
Scenedesmus rubescens dry extract showed no photoprotection at effective concentrations
To exclude photoprotective effects of the Scenedesmus rubescens dry extract by absorbance, we analysed the absorbance spectrum of a 1% and a 0.1% solution of the dry extract (Fig. 1). We found minimal absorbance in the UVR spectral region.

Scenedesmus rubescens extract boosted viability of dermal fibroblasts in vitro
We found that Scenedesmus rubescens extract was able to increase basal dermal fibroblast viability as assessed by MTT test in a dose-dependent and significant manner up to 127% ($P < 0.05$) (Fig. 2a) suggesting enhanced mitochondrial activity. In addition, we irradiated dermal fibroblasts with UVA (20 J/cm²). When we applied the Scenedesmus rubescens dry extract 1 h before irradiation, we found a trend towards improved viability of dermal fibroblasts up to 156% with optimal efficacy of 0.00001% dry extract (Fig. 2b). Applying the dry extract 1 h after UVA irradiation resulted in a dose-dependent and significant improvement of cell viability up to 304% ($P < 0.05$) at 0.001% dry extract (Fig. 2c).

Scenedesmus rubescens dry extract boosted collagen content in dermal fibroblasts in vitro
Incubation of the Scenedesmus rubescens dry extract with dermal fibroblasts for 48 h led to a dose-dependent and significant increase in total collagen content as assessed by picrosirius red staining up to 134% ($P = 0.07$) at 0.001% dry extract (Fig. 3).

Scenedesmus rubescens dry extract inhibited mushroom tyrosinase activity in vitro
It has been shown that photoageing leads to uneven skin tone and hyperpigmented spots (age spots) (Hillebrand 2001). Therefore, inhibition of tyrosinase, the rate-limiting enzyme in melanogenesis, would be beneficial in the prevention of age-related cutaneous discoloration [21]. We found that the Scenedesmus rubescens extract was able to inhibit the activity of tyrosinase in vitro (Fig. 4). It may therefore act against hyperpigmentation in photoaged skin.

Scenedesmus rubescens extract stimulated collagen III ex vivo
Furthermore, we investigated the efficacy of the Scenedesmus rubescens extract in skin tissue. We tested its collagen-stimulating...
activity by measuring collagen III content in skin after topical treatment with the extract. Collagen III decreases with age [22], and we found that the *Scenedesmus rubescens* extract was able to significantly increase collagen III content in skin tissue *ex vivo* (Fig. 5). The stimulation at 1% dry extract was 129% (*P* < 0.05). This confirmed the results of Fig. 3 where stimulation of total collagen in dermal fibroblasts after treatment with *Scenedesmus rubescens* dry extract was shown.
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Figure 5 *Scenedesmus rubescens* dry extract significantly and dose-dependently stimulated collagen III synthesis when applied topically *ex vivo*. Retinoic acid 0.05% was used as positive control to validate the assay. Means with standard error of the mean are shown. *P < 0.05, **P < 0.01 vs vehicle control by unpaired t-test.

Figure 6 *Scenedesmus rubescens* dry extract was able to prevent the formation of sunburn cells significantly and dose-dependently when applied topically *ex vivo*. A commercial standard sunscreen (SPF 50+) was used as positive control to validate the assay. Means with standard error of the mean are shown. ***P < 0.001, **P < 0.01 both vs vehicle UVB, both by unpaired t-test.

*Scenedesmus rubescens* extract protected from sunburn cell formation *ex vivo*

One easy-to-monitor damage caused by UVB irradiation is the formation of sunburn cells in skin [15]. We found that the *Scenedesmus rubescens* extract protected the skin from formation of UVR-induced sunburn cells *ex vivo* by up to 37% (*P < 0.01) at 1% use level (Fig. 6). Sunburn cells [15] are UVR-damaged cells going into apoptosis. The extract even outperformed a standard commercial sunscreen with an SPF of 50+.

Discussion

Solar irradiation is still considered the main reason for cutaneous ageing. Within the solar spectrum, UVR has been extensively investigated and its damaging effects in skin, from cosmetically relevant signs such as wrinkles, sagging and age spots to pathological conditions such as atopic keratoses or skin cancer, are well documented. Using sunscreens containing filters against both UVB and UVA radiation represents the first and main line of defence against cutaneous damage (nicely reviewed in Young *et al.* [23]). However, from a skincare perspective, it would be desirable to have in addition a skincare active that could strengthen the skin’s metabolism in order to fight the damaging effects of solar irradiation. The results obtained with an extract from the freshwater microalga *Scenedesmus rubescens* presented in this report show a broad spectrum of action against ageing attributed to UVR. In particular, we found that the extract can increase dermal fibroblast viability as assessed by MTT assay, meaning that mitochondrial activity is boosted (Fig. 2a). This increased viability was also seen when the cells were irradiated with UVA, providing evidence for protection against UVA-induced cellular damage (Fig. 2b and c). We obtained an increase of up to 3 times more mitochondrial activity. Although not directly tested, this often goes along with an increase in proliferation. A three-fold increase in proliferation may seem high and raises questions whether this is good or bad. As this was an *in vitro* experiment with isolated cells, this may not have the same impact on whole skin. We therefore think that there is an increased activity, but we would not so much rely on the actual numbers which may not translate to an *in vivo* situation. Decreased dermal collagen is one of the hallmarks of photoageing [9] leading to wrinkle formation and loss of firmness. Particularly, collagen III has been shown to decrease significantly with increasing age [22, 24]. The extract of *Scenedesmus rubescens* was able to increase total collagen *in vitro* (Fig. 3) and boost collagen III *ex vivo* (Fig. 5). We have not tested this in relation to solar irradiation. However, cellular viability was strengthened both in the absence and in the presence of UVA (Fig. 2). Hence, we hypothesize that the same will be true for the collagen-synthesizing activity. We additionally found good protection against UBV-induced sunburn cell formation *ex vivo* (Fig. 6) outperforming even a standard commercial sunscreen with an SPF of 50+. These data demonstrate that the extract has a broad spectrum of activity against ageing attributed to UV irradiation. It was previously shown that the action spectrum for sunburn cells was most efficient in the UVC range; however, using high doses of wavelengths above 290 nm also induced sunburn cells [25]. In addition, the skin tissue we used was from a rather darkly pigmented Caucasian donor. This is why we had to administer a seemingly high UVB dose of 1 J/cm² in order to achieve sunburn cell formation. Looking at the absorbance spectrum of the *Scenedesmus rubescens* extract, we found that a solution containing 0.1% extract had only marginal absorbance in the UVA and UVB radiation spectrum (between 290 and 400 nm) (Fig. 1). Based on the Beer–Lambert law [26], we conclude that, as the tests in Fig. 2 were carried out with concentrations below 0.001%, the protective effects found were due to biological activity and not UVR absorbance. Along these lines, we also propose that the protective effects against formation of sunburn cells was due to biological activity, although here, as we used concentrations of 0.1% and...
1%, a contribution due to UVB absorbance cannot completely be ruled out. The composition of this extract of *Scenedesmus rubescens* is rich in amino acids essential for the building of skin collagen such as proline, alanine and glycine [27], as well as the precursor of proline glutamic acid (not shown). Particularly, the supplementation of proline was shown to aid in the cutaneous collagen recovery after UV irradiation in mice [28]. Furthermore, it contains niacin (vitamin B3) which is an important energy storage and proven anti-ageing active [29]. We propose that the extract composition rich in these factors contributes to strengthening the skin against the impact of solar irradiation.

In summary, we provide evidence that an extract of the freshwater microalga *Scenedesmus rubescens* has broad anti-ageing activity against UV irradiation-induced cutaneous damage.

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**Conflict of interests**

This study was funded by DSM Nutritional Products. The extract of the microalga *Scenedesmus rubescens* mentioned in this manuscript is marketed by DSM Nutritional Products under the trade name PEPHA®-AGE. The authors report no other conflict of interests.