Repair of UVB-Damaged Skin by the antioxidant sulphated flavone glycoside Thalassiolin B

Isolated from the marine plant *Thalassia testudinum* Banks ex König

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Abstract

Daily topical application of the aqueous ethanolic extract of the marine sea grass, *Thalassia testudinum*, on mice skin exposed to UVB radiation resulted in a dose dependent recovery of the skin macroscopic alterations over a 6-day period. Maximal effect (90%) occurred at a dose of 240 $\mu\text{g}/\text{cm}^2$, with no additional effects at higher doses. Bioassay-guided fractionation of the plant extract resulted in the isolation of thalassiolin B (1). Topical application of 1 (240 $\mu\text{g}/\text{cm}^2$) markedly reduces skin UVB-induced damage. In addition, thalassiolin B scavenged 2,2-diphenyl-2-picrylhydrazyl radical with an $\text{EC}_{50}=100 \mu\text{g}/\text{ml}$. These results suggest that thalassiolin B is responsible for the skin regenerating effects of the crude extract of *T. testudinum*.

Introduction

Considerable exposure to ultraviolet (UV) light can have extremely noxious effects on skin. Specifically, UVB acute exposure to the skin induces direct and indirect adverse biological effects. By impairing oxidative balance the oxidative stress (Inal et al., 2001) results in actinic erythema, sunburn, photo-induced diseases, photo-worsened diseases, actinic ageing and skin cancers (Kligman et al., 1984; Saliou et al., 2001; Hwang et al., 2006; Svobodova et al., 2006). One approach to protecting humans from the harmful effects of UV irradiation is to use antioxidative phenolic compounds (Svobodova et al., 2003). Several lines of evidence have shown that phenolic plant compounds such as caffeic and ferulic acids, quercetin and apigenin can reduce the harmful effects of UV radiation (Birt et al., 1997; Saija et al., 2000; Inal et al., 2001). Although investigations are scarce, some authors have demonstrated that seaweed extracts can help to protect skin from UV radiation (Fujimura et al., 2002; Bulteau et al., 2006).

Thalassia testudinum a sea grass, commonly known as turtle grass (Littler and Littler, 2000), plays an important role in marine ecosystems (Durako et al., 2002), supplying nursery grounds for many invertebrate and fish species, stabilizing the sediments of near shore coast (Corlett et al., 2007), etc. Previous studies have shown that luteolin 7- β -D-glucopyranosyl-2"-sulphate (thalassiolin A), isolated from *T. testudinum* (collected in the Bahamas coasts) has antibiotic activity against *Zoosporic fungi* (Jensen et al., 1998). Moreover, another study demonstrated that thalassiolins A-C represent a new series of HIV integrase inhibitors (Rowley et al., 2002).

In the present work, we describe the effect of the topical application of a cream containing the aqueous-ethanolic extract of *T. testudinum* leaves ($60 \mu\text{g}/\text{cm}^2$ - $240 \text{mg}/\text{cm}^2$) on mice undergoing acute UVB skin damage. By means of a bioassay-guided fractionation of the active extract we isolated and purified thalassiolin B (2) as the major metabolite present. In addition, 4-hydroxybenzoic acid (1) was isolated for the first time from this plant. The structures of 1 and 2 were elucidated by spectroscopic analysis (^1H and ^{13}C NMR, gCOSY60, gHSQC, gHMBC and ROESY experiments and MS measurements) and compared to the literature (Jensen et al., 1998; Rowley et al., 2002).

Results

Repair of Acute UVB-damaged Skin by the extract. We have previously shown that there was no evidence of skin irritation or toxicity from repeated crude extract cream applications. Exposure to UVB radiation results in the development of macroscopic alterations such as: erythema, scabs, roughness and wrinkling of the skin in irradiated control animals. The peak of reaction occurred between 48 and 72 h in all animals. However, topical application of crude extract of *T. testudinum* leaves resulted in dose-dependent reduction of the macroscopic alterations (Fig. 1). The maximal effects occurred at 240 $\mu\text{g}/\text{cm}^2$, with no additional effect observed at higher concentrations (up to 480 $\mu\text{g}/\text{cm}^2$). Skin damage was significantly suppressed by 90% (mean value) at the end of the application period. The significant inhibitory effect of the crude extract at the maximal inhibitory dose on skin damage was further confirmed by histopathological studies (Fig. 2 and 3). As observed, non treated mice skin showed severe histopathological alterations such as acanthosis, hyperkeratosis, infiltrating inflammatory cells, collagen and elastic fibres degradation. In addition, macrophages were found in higher numbers in the photodamaged skin of untreated animals. However, for animals treated topically with a cream containing crude extract, the erythema was eliminated, collagens and elastics fibres were reorganized, acanthosis and hyperkeratosis decreased, and vascular damage was also reduced.

Bioassay Guided Fractionation and Identification of Active Compound. The crude extract (48 g) was dissolved in water and successively partitioned with *n*-hexane (Fraction H, 50 mg), chloroform (Fraction C, 240 mg), and water-saturated *n*-butanol (Fraction B, 800 mg). The residual aqueous fraction was lyophilized (Fraction A, 46 g) and further fractionated by precipitation with EtOH (95 %), the soluble fraction was designated fraction S (10 g) and the residue as fraction N (35 g). All fractions were dried and tested for skin regenerating activity. As shown in figure 1 fraction B and S exhibited skin regenerating activity as judged by significant reduction of macroscopic signs. Fraction B showed the most potent activity, reducing macroscopic skin alterations to the same extent as the crude extract.

Results of the antioxidant activity and phenolic content of the crude extract and fractions B and S are shown in table 1. The results indicate that each of the fractions exhibit radical trapping

properties against DPPH radical. Fraction B exhibited the strongest scavenging activity with an EC_{50} of 97 $\mu\text{g/mL}$. In addition, fraction B showed the highest phenolic content. Extract B was then fractionated by size exclusion column chromatography (Sephadex LH-20, $\text{Cl}_3\text{CH}:\text{MeOH}$, 5:1) to give a late eluting yellow band that was further fractionated via silica gel column chromatography (using 7:3:0.25 $\text{Cl}_3\text{CH} : \text{MeOH} : \text{H}_2\text{O}$ as eluent). This was followed by C18 reversed phase HPLC purification (linear gradient 5 to 30% $\text{MeOH}/\text{H}_2\text{O}$ in 40 min) to give pure thalassiolin B (2) (40 mg). In addition, a 15 mg fraction containing 4-hydroxybenzoic acid (1) was also isolated from the LH20 chromatography. Pure 1 was obtained after silica gel column chromatography using 2:1 $\text{Cl}_3\text{CH} : \text{AcOEt}$ as eluent.

4-Hydroxybenzoic acid (1) was isolated as white crystalline solid and with an $[\text{M}-\text{H}]^-$ ion at m/z 139 in the ESIMS. The structure was confirmed by analysis of ^{13}C and ^1H NMR chemical shifts. Thalassiolin B (2) (chrysoeriol 7- β -D-glucopyranosyl-2"-sulphate) was isolated as yellow amorphous solid with an $[\text{M}-\text{H}]^-$ ion at $m/z = 541$ in the ESIMS. The structure was confirmed by spectroscopic analysis (^1H and ^{13}C NMR, gCOSY60, gHSQC, gHMBC and ROESY experiments) and compared to the ^1H - and ^{13}C -NMR spectra data with the previously reported values (Jensen et al., 1998; Rowley et al., 2002).

Compounds 1 and 2 were tested for skin regenerating activity. The results (Fig 1) show that serial administration of thalassiolin B markedly restored skin damage induced by acute UVB radiation exposure by significantly reducing macroscopic alterations. Thalassiolin B exhibits stronger activity than the crude extract and by the end of the experiment (6 days) almost all of the topically-treated UVB-induced damaged skin (99 %, $N = 10$) had recovered, according to the macroscopic parameters examined, to the extent that there were no significant differences between the control and the thalassiolin B treated group. Histopathological examination (Fig 2 and 3) confirmed the macroscopic observations. Thalassiolin B treated skin exhibited little difference compared to non-irradiated skin and suggests that the skins of mice, treated with this compound, were recovered to within the normal range over the treatment period. The elastic fibre content was within normal range, no detectable loss of collagen fibre was observed and no acanthosis and inflammatory infiltrates were observed. When 4-hydroxybenzoic (1) was applied topically no skin regenerating

activity was seen (Fig 1).

Thalassiolin B and 4-hydroxybenzoic acid were assayed for radical trapping activity. Thalassiolin B was found to scavenge DPPH radicals with an $EC_{50} = 100.0 \mu\text{g/mL}$. However, 4-hydroxybenzoic acid was found not scavenge this radical at concentrations $> 400 \mu\text{g/ml}$ (Table 1).

Discussion

Several lines of evidence have shown that UVB-induced acute photodamage is characterized by degradation of collagen and accumulation of abnormal elastin in the superficial dermis. UV radiation causes collagen to break down at a higher rate than with just chronologic aging (Fisher et al., 1996; 2002). Mast cells and macrophages, which are found in higher numbers in photoaged skin, have been implicated in this process. Skin inflammation further traumatizes the damaged skin, which causes bleeding into the affected tissues, such that haemoglobin is released from the red blood cells (Kaminer 1995; Trautinger, 2001). Our current results agree with these facts, while macroscopic alterations occurred in all the animals after UVB acute radiation. Morphological study demonstrated that control irradiated animals showed an increase in damaged collagen fibres, skin thickness, hyperkeratosis and infiltrated mononuclear cells at the end of the experimental period.

However, macroscopic observations showed that the UVB-damaged skin that appeared over a 48-72 h period in all animals was gradually repaired during the continued application of the crude extract of *T. testudinum* leaves. In agreement with this, histopathological studies revealed the presence of new connective tissue with parallel collagen bundles and a network of fine elastic fibres at the end the experimental period. Besides, a decrease in mononuclear cells was appreciated. Accordingly, our present results suggest that the continuous topical application, post irradiation, of the crude extract of *T. testudinum* leaves repairs UVB-induced acute mice skin damage, probably by stimulating the skin regeneration processes. This hypothesis is consistent with observations that the new collagen formed in the repair zone resembled that of the normal non irradiated tissue with respect to the staining properties. It should be noted that the presence of fibroblasts, the major collagen-producing cells in skin, was also increased having morphological features characteristic of high metabolic rate. These results suggest that the crude extract can contribute to skin repairing by increasing synthesis of collagen fibres by hyperactive fibroblasts.

Our results also showed that fraction S and B exhibited skin regenerating effects. Fraction S significantly inhibited macroscopic skin alteration by about 80 % and fraction B by about 90 %, when were applied at the same dosage range than the extract. It is interesting to note that fraction B, the most active fraction, also exhibited the strongest radical scavenged effect against DPPH as well as the higher content of phenolic structures. Other authors have previously demonstrated the radical trapping properties and the antilipoperoxidative action of phenolic structures (Rice-Evans et al., 1996; Rice-Evans 2001; Nakai et al., 2006). Also, it has been demonstrated that pro-oxidant states may contribute to initiate UV-induced damage (Inal et al., 2001; Fuchs et al., 2001) and that UV acute exposure deplete antioxidant enzymes and induce protein oxidation in skin (Sander et al., 2002; Pillai et al., 2005; Sin et al., 2005) that impair the skin's ability to protect itself against the free radicals generated after exposure to UVB radiation. Thus, our results suggest that the skin regenerating effects of the crude extract may be due in part by its antioxidant effects of the phenolic structures.

Purification steps of the active fraction B allows the isolation of thalassiolin B and 4-hydroxybenzoic acid as major components. However, the skin regenerating activity was solely observed for the topical application of thalassiolin B, showing an increased magnitude of skin regenerating activity as that presented by the crude extract. Moreover, thalassiolin B also showed antioxidant capacity, which was stronger than that observed by the crude extract. Thus, our results suggest that the skin repairing activity of crude extract may be due to the presence of thalassiolin B and that its antioxidant effect may partially account for its skin regenerating activity.

Histological examination of skin after thalassiolin B treatment resembles those found for the crude extract, revealing that in irradiated topically-treated skin all parameters, observed with Hematoxylin and Eosin stain, were within the normal ranges. Also, collagen damage, as judged by loss of affinity for the 'Van Gieson' stain, was virtually nonexistent and an increased number of fibroblast was observed, suggesting areas of new collagen synthesis. Thus, our results suggest that the skin of irradiated mice was capable of displaying a wide range of responses to topical application of thalassiolin B, given rise to skins that appeared similar to that of the control group.

Previous results have shown that luteolin 7- β -D-glucopyranosyl-2"-sulphate (thalassiolin A), a very structurally closed flavone isolated from *T. testudinum*, has antibiotic activity against *Zoosporic fungi* (Jensen et al., 1998). Also, was demonstrated that thalassiolins A, B and C (apigenin 7- β -D-glucopyranosyl-2"-sulphate) inhibit, in different degree, HIV integrase protein (Rowley et al., 2002). In our study we have demonstrated, for the first time, the antioxidant and skin regenerating activity of thalassiolin B. Thus, as occurred with other phenolic structures (Widyarini et al., 2001; Dajas et al., 2003; Tasdemir et al 2006; Choi et al., 2007; Ogawa et al., 2007) thalassiolin B also exhibits several pharmacological activities, raising the possibility of an effect on several mechanism of skin regeneration. However, whether or not thalassiolin B may affects other step of the cytotoxic cascade induced by UV radiation deserves further investigations.

Finally, our present results also showed that fraction S inhibited skin damage when evaluated in the same manner as fraction B. Thus, the contribution of other components of the extract will be settling after the identification and characterization of the constituent of this fraction.

Experimental

General Experimental Procedures. Column chromatography was carried out using Silica gel 60 Merck (35-70 and 70-230 mesh), Sephadex LH-20 (Pharmacia, Sweden). Silica gel 60F254 TLC plates were used to check the purity of compound 1. TLC plates were visualized under UV light (254 and 365 nm) with a Vilber Lourmat lamp and subsequently sprayed with perchloric acid / vainillin (1 %) on the TLC. K-1001 Knauer pump equipped with UV K-2501 detector and a Bondapack Waters C18 (10 x 250 mm) column were used for HPLC. All solvents used for HPLC were Fisher HPLC grade. Melting point was determined on an Electrothermal-9200 melting-point apparatus. All NMR experiments were performed at ambient temperature on a Bruker AV-600 MHz spectrometer (TMS as internal reference) in CDCl₃ + CD₃OD (1) and DMSO-d₆ (2). Low resolution ESI-QIT-MS were recorded on a Bruker-Hewlett Packard 1100 Esquire-LC system mass spectrometer in negative mode (2). The EI mass spectrum of 1 was recorded on a MS Concept I/HQ mass spectrometer. UV absorption spectra were recorded on a Shimadzu UV-1201 spectrophotometer. Optical rotation was measured using a Jasco P-1010 Polarimeter with sodium light (589 nm).

Sea grass Collection. *T. testudinum* Banks & Soland ex. Koenig was collected in March 2004 from “La Concha” Beach (22°05'45"N 82°27'15"W) and identified by Dr. Areces J.A. (Institute of Oceanology, Havana, Cuba). A voucher sample (No. IdO39) has been deposited in the herbarium of the Cuban National Aquarium.

Extraction and Isolation. Whole dry and ground *T. testudinum* leaves (8 kg) were continuously extracted with EtOH / H₂O (50:50, vol/vol) during 7 days at room temperature. The combined extracts were filtered and concentrated under reduced pressure and low temperature (40 °C) to yield 48 g of residue. The bioassay-guided chemical fractionation of the crude extract (explained in the results part) led to the isolation and identification of compounds 1 and 2.

Measurement of Antioxidant Activity

2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The antioxidant activity of the crude extract, fractions B and S, and compounds 1 and 2 was measured in terms of radical-scavenging ability according to DPPH method of Brand-Williams (1995) with minor modifications. Basically, a 60 µM methanolic solution of DPPH (980 µl) [Sigma-Aldrich Co. (St. Louis, MO)], daily prepared, was placed in a spectrophotometer cuvette, and different concentrations of samples in DMSO: MeOH (v/v) solution (20 µl) were added. The decrease in absorbance at 515 nm was determined at 1 min intervals, in a UV-1201 spectrophotometer (Shimadzu, Japan) with Data Capture software, until the reaction plateau step was reached; a time of 180 min was needed to arrive to this plateau step with all samples. Methanol was used to adjust zero the spectrophotometer. EC₅₀ values were determined from the plotted graph of scavenging activity against the concentration of samples. Triplicate measurements were carried out, and their scavenging effect was calculated based on the percentage of DPPH scavenged.

Total polyphenols content. Total phenolic content was determined according to the method described in the British Pharmacopeia (2007). The samples were extracted with 2 ml of distilled water at room temperature for 2 h and was centrifuged at 1000g for 15 min. The supernatant (5 ml) was mixed with water to 25 ml. This solution (2 ml) was mixed with 1 ml of Folin-Ciocalteu's phenol reagent, 10 ml of water and a 29.0% w/v solution of sodium carbonate to 25 ml. After 30 min, absorbance was read at 760 nm. Results were expressed as pyrogallol (1,2,3-Trihydroxybenzol)

equivalents (Merck). The concentration used was in a range between 0.005 and 0.099 mg/ml.

Animals and Treatment. Animal care and experimental procedures were done in accordance to the Helsinki Declaration. The number of animals used in the present experiments was kept to a minimum required for meaningful interpretation of the data. Male albino mice (Balb/C, 25 ± 1.5 g) obtained from the Centre for Animal Lab Production (Havana, Cuba) were used. Before the experiments, animals were adapted to laboratory conditions with water and food *ad libitum*. Prior to the assay, mice were depilated in the back (2 cm^2). One day after depilation, the animals were divided into the experimental groups composed of three control groups, of ten animals each: an irradiated control, an irradiated control treated topically with vehicle and the irradiated group treated with an antiaging cream containing natural extracts (Ultra Facial, UF, Zermat Internacional S.A). In the treated group, crude extract of *T. testudinum* leaves was topically applied at different concentrations. The base cream used as vehicle was a simple oil-in-water cosmetic emulsion prepared without preservatives and prepared by adding the same volume of distilled water. The crude extract and fractions were dissolved in distilled water and mixed with this base cream by manual agitation to produce creams (0.008, 0.004, 0.002 and 0.001 mg of crude extract /mg of cream (wt/wt) and 0.004 mg of the fractions /mg of cream). Weighed aliquots of the cream were applied by spreading evenly over the dorsal area with a plastic applicator (60 mg/cm^2). Mice were held on their cage tops for approximately 15 min while the cream was absorbed to discourage loss of cream by contact with the cage or bedding.

Experimental Design. Animals were exposed to ultraviolet radiation (UVB) (312 nm) with a Spectroline® lamp (Spectronics Corporation, New York, U.S.A). Irradiance was at the back of the mice (30 cm from the lamp) for an interval of 6 min., immediately after exposure, animals were treated and, later on, at intervals of 24 h during 6 days. Daily, before each treatment, animals were observed by the same observer in order to detect the presence or not of macroscopic skin alterations. Dermal alterations, such as; erythema, scabs, roughness and wrinkling of the skin, were quantified in a 1 to 4 point value. At the end of the experiment, 24 h after the last treatment, mice were euthanized and the skins were collected for histological analysis.

Histological Analysis. Six animals per group were analyzed. Dorsal skins biopsy specimens, 2 cm

were processed for light microscopy. The samples were fixed in 10% buffered formalin for 24 h, processed routinely, and embedded in paraffin. Slices of 5 µm were used for the analyses and stained with Hematoxylin and Eosin and Van Gieson's for collagen. The slides were evaluated in coded manner.

Statistical Analysis. Statistical analyses were done by using the statistical package SPSS V.10.0 2000 for Windows. Comparisons between control and treated groups were done by the Mann Whitney U-test. A two tailed test was used and $P < 0.05$ was considered to be statistically significant.

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Table 1. Scavenging activity (EC₅₀) on DPPH radicals and phenolic content (%) of samples from *T. testudinum*

Samples	DPPH Scavenged (EC ₅₀) ^a	Total phenolic content ^b
crude extract	158 µg/ml	18 ± 1.5 %
fraction B	97 µg/ml	39 ± 1.5 %
fraction S	360 µg/ml	15 ± 2.1 %
4-hydroxybenzoic acid	> 400 µg/ml	-
thalassiolin B	100 µg/ml	-

^aValues are expressed as mean ± standard deviation (n = 3). Coefficients of variance (CV) are less than 20%.

^bResults are expressed as phyrogallol equivalents. Values are also expressed as mean ± standard deviation (n = 3).

Figure 1

Figure 2

Figure 3

Figure 4

Fig. 1. Effects of the samples in photodamaged skins. Irrad (irradiated), PI (vehicle), UF (antiaging cream), E1 (crude extract, 240 $\mu\text{g}/\text{cm}^2$), E2 (crude extract, 120 $\mu\text{g}/\text{cm}^2$), E3 (crude extract, 60 $\mu\text{g}/\text{cm}^2$), B (fraction B), S (fraction S), TB (Thalassiolin B) and 4-PHB (4-hydroxybenzoic acid) (B, S, TB and 4-PHB, 240 $\mu\text{g}/\text{cm}^2$).

Fig. 2. Hematoxylin and Eosin stain (X10 magnification) (A) Untreated skin: the normal epidermis. (B) Photodamaged skin: acanthosis marked, hyperkeratosis, infiltrating inflammatory cells. (C) Topically treated with crude extract and (D) Topically treated with Thalassiolin B: the skin is comparable to A, there is an increment of fibroblast and epidermal regeneration.

Fig. 3. Van Geison's stain (X25 magnification). (A) Untreated skin: the normal epidermis. (B) Photodamaged skin: acanthosis marked, hyperkeratosis, infiltrating inflammatory cells, collagen alterations. (C) Topically treated with crude extract and (D) Topically treated with Thalassiolin B: epidermal regeneration, reorganized collagen fibers.

Fig. 4. Structures of compounds 1 and 2 isolated from *T. testudinum*