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PHYTOCHEMICAL PROFILE AND EVALUATION OF PHOTOPROTECTIVE POTENTIAL OF *Syringodium filiforme* KÜTZING

PERFIL FITOQUÍMICO Y EVALUACIÓN DEL POTENCIAL FOTOPROTECTOR DE *Syringodium filiforme* KÜTZING

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ABSTRACT

Syringodium filiforme Kützing (Cymodoceaceae) is a marine seagrass abundant in Caribbean Sea, rich in phenolic compounds which have antioxidant properties and can provide new opportunities for treatment and prevention of diseases mediated by ultraviolet radiation like photoaging and skin cancer. The aim of this study was to evaluate the phytochemical profile and the photoprotective potential of *S. filiforme* leaf extracts . Total phenolic and flavonoid contents were 72.85 ± 0.72 mg pyrogallol equivalents/g dry extract (PE) and 59.09 ± 0.45 mg quercetin equivalents/g dry extract (QE), respectively. The total anthocyanins content was 1.35 ± 0.02 mg malvidin-3-O-glucoside equivalents/g dry extract (ME). The extract showed photoprotector potential in the UVB region. The *S. filiforme* treated mice showed a significantly decreased wrinkling score, and a reorganization of the collagen fiber was observed compared with irradiated and not treated skin. These results suggest that the crude extract of *S. filiforme* leaves may be a promising natural sunscreen product.

Keywords*: Syringodium filiforme* Kützing, ultraviolet, photoprotective, chronic skin damage.

RESUMEN

Syringodium filiforme Kützing (Cymodoceaceae) es una planta marina abundante en el Mar Caribe, rica en compuestos fenólicos, que tiene propiedades antioxidantes y puede proporcionar nuevas oportunidades para el tratamiento y prevención de enfermedades mediadas por radiación ultravioleta como el fotoenvejecimiento y el cáncer de piel. El objetivo de este estudio fue evaluar el potencial fotoprotector del extracto de hojas de *S. filiforme*. El contenido de fenoles totales y flavonoides fue de 72,85 ± 0,72 mg equivalentes de pirogalol / g de extracto seco (PE) y 59,09 ± 0,45 mg de equivalentes de quercetina / g de extracto seco (QE), respectivamente. El contenido de antocianinas totales fue de 1,35 ± 0,02 mg equivalentes de malvidin-3-O-glucósido / g de extracto seco (ME). El extracto mostró potencial fotoprotector en la región UVB. Los ratones tratados con *S. filiforme* mostraron una puntuación de arrugas significativamente disminuida, y se observó una reorganización de la fibra de colágeno en comparación con la piel irradiada y no tratada. Estos resultados sugieren que el extracto crudo de hojas de *S. filiforme* pudiera ser un promisorio protector solar natural.
Palabras clave: *Syringodium filiforme* Kützing, ultravioleta, fotoprotector, daño crónico de la piel.

INTRODUCTION

Solar ultraviolet (UV) exposure is one of the most important environmental factors affecting skin physiology. Various skin disorders, such as wrinkling, scaling, dryness and pigment abnormalities can be initiated by exposure to solar UV radiation, which has been reported by various clinical, epidemiological and biological studies to be the major etiological agent in the development of skin cancers (Lo & Fisher, 2014; Bowden, 2004).

Skin contains antioxidant defenses, but these will be overwhelmed if the dose of UV light is high enough, and this results in free radical damage to cellular components. Most dermatologists agree that antioxidants help fight free radical damage and can help maintain healthy skin. They do so by affecting intracellular signaling pathways involved in skin damage and protecting against photodamage, as well as preventing wrinkles and inflammation (Oresajo *et al*., 2012).

In recent years there have been an increment in studies for applications of marine bioactive compounds. At present it is known that various commercial nutritional supplements prepared from extracts of seaweeds are of great importance for the health care and protection against age-related diseases (Pallela *et al*., 2010; Zhao & Chen, 2014).

Recently, marine originated photoprotective or anti-photoaging behavior was observed in the methanol extracts

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of *Corallina pilulifera*. These extracts were found to exert potent antioxidant activity and protective effect on UVA-induced oxidative stress in human dermal fibroblast cells, by protecting DNA and also by inhibiting matrix metalloproteinases (MMPs) (Ryu *et al*., 2009). In recent studies Fucoxanthin, a characteristic carotenoid present in edible brown seaweeds, showed great antioxidant activity, anti-cancer, anti-diabetic and anti-photoaging properties (D'Orazio *et al*., 2012). From an ethyl acetate fraction of *Zostera marina* L., luteolin was isolated, which possesses anti-skin photoaging effect through inhibition of MMP-1 (Kim *et al*., 2004). Aaptamine, an alkaloid isolated from the sponge *Aaptos suberitoides*, has been reported to attenuate the expression of MMPs in UVB-irradiated human dermal fibroblasts. Aaptamine also decreased proinflammatory cytokines such as cyclooxygenase-2, tumor necrosis factor-α, interleukin-1β, and nuclear factor-kappa B subunits in UVB-irradiated human keratinocytes (Kim *et al*., 2014). In a previous work, our research group reported that the topical application of thalassiolin B, a sulphated flavone glycoside isolated from *Thalassia testudinum*, shown skin-regenerating effects (Regalado *et al*., 2009).

As it is evident that unregulated expression of MMPs leads to photoaging, many research groups are emphasizing their research goals to check the ability of marine-derived phlorotannins as potential anti-photoaging agents (Wijesinghe and Jeon, 2011).

Syringodium filiforme Kützing (Cymodoceaceae), a marine seagrass abundant in the Caribbean Sea, is a matter of great importance to the cosmetic and food industries. From the methanolic and aqueous extracts of this specie Grignon-Dubois and coworkers (Nuissier *et al*., 2010) isolated chicoric and caftaric acids as major polyphenols. Recently, González-García and coworkers (2011) found in the methanolic fraction, which showed significant free radical scavenging properties, high concentrations of flavonoids, phenols and antocyanins. The ability of *S. filiforme* Kützing to absorb UVB rays was determined by UV/visible light spectrophotometry (González-García *et al*., 2011). *S. filiforme* showed a high absorptive capacity in the range of UVB light (280–320 nm), with peak positions at 278 and 312 nm. In this context, *S. filiforme* could represent a novel and effective strategy for treatment and prevention of photoaging.

Considering the lack of studies on the photoprotective properties of the extract of *S. filiforme* leaves, the aim of this study was to evaluate its photoprotective potential. To the knowledge of the authors this is the first study showing prevention of UV-mediated damages in skin by *S. filiforme*.

MATERIALS AND METHODS

Plant materials

S. filiforme leaves were collected from Guanabo Beach (23º10'44"N - 82º07'01" W) Havana, Cuba, in March 2012. A voucher specimen was authenticated by Dr. A. J. Areces, Institute of Oceanology, Havana, Cuba. The voucher was deposited in the collection of the National Aquarium from Cuba, with number IDO 165. The collected seagrass was washed with distilled water to remove sand and salts and then dried in an oven at 50 °C to constant weight.

Extraction and fractionation

Dried and powdered leaves of *S. filiforme* (200 g) were extracted with a mixture of ethanol: water (2 L, 1:1 v/v) at room temperature for 1 week, filtered and concentrated to dryness to yield the total extract (5.05 g). Four grams of the total extract were fractionated with help of mechanic agitation with *n*-hexane (40 mL), chloroform (40 mL), *n*-butyl alcohol (40 mL) and water (40 mL), to yield *n*-hexane, chloroform, *n*-butanol (0.177, 1.255, 0.262 g, respectively), and H_2O-soluble (1.627 g) fraction.

Preliminary phytochemicals studies

Quantification of metabolite families was performed by standard phytochemical reaction methods using UV detection: total polyphenols (British Pharmacopeia 2009), flavonoids (Woisky & Salatino, 1998), total anthocyanins (Fuleki & Francis, 1968), and total carbohydrates by phenol-sulfuric methods (Dubois *et al*., 1956), chlorophylls (Wrolstad *et al.*, 2005) and soluble proteins (Bradford, 1976).

Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) assay whereby the tetrazolium salt, MTT is reduced by intracellular dehydrogenases of viable living cells leading to the formation of purple formazan crystals. Following UV-exposure, cells were washed twice with PBS and incubated in the presence of MTT salt solution at a concentration of 0.5 mg/mL for four hours at 37 ºC. The medium was removed and the crystals were dissolved in DMSO. The optical density of each well was read at 540 nm using a microplate reader (Gómez-Lechón *et al.,* 2003). Cell viability was expressed as a percentage of live cells compared to unexposed control.

Animals

The experimental procedure that involved the use of animals was approved by the Animal Ethics Committee of the Centre of Marine Bioproducts. Adult male albino mice, weighing approximately 25 g (Balb/C, 25 ±1.5 g), were obtained from the Center for Animal Lab Production (Havana, Cuba). Animals were maintained in individual cages on a 12:12 h light–dark cycle in a temperature-controlled room, with access to water and food ad libitum until use.

Irradiation and S. filiforme treatment

The UV apparatus consisted of a Spectroline® lamp (Spectronics Corporation, New York, USA). The spectral irradiance for the UV lamps was 312 nm, providing 100% UVB. The irradiation was made at 30 cm from the dorsal surface of the mice. Prior to the assay, mice were depilated in the back (2 cm^2). The vehicle or base cream used was a simple oil-in-water cosmetic emulsion without preservatives and this is prepared by adding the same volume of distilled water. The

crude extract and fractions were dissolved in distilled water and mixed with this base by manual agitation to produce creams. Immediately after exposure, animals were treated at 24 h-intervals for 7 days. At the completion of the experiment, mice were sacrificed by cervical dislocation and the skins were collected for histophatological study. A total of 60 mice (10 mice/group) were divided into: (a) Irradiated (irradiated without treatment), (b) placebo (irradiated and treated topically with vehicle), (c) Positive Control (irradiated and treated with an antiaging cream containing natural extracts, Ultra Facial (UF), Zermat International S.A), (d) Total extract (irradiated and topically treated with extract of *S. filiforme*), (e) Aqueous fraction (irradiated and treated topically with the corresponding aqueous fraction), f) chloroform fraction (irradiated and treated topically with the chloroform fraction). Treatments were applied evenly to the skin of the back at a rate of at 240 µg/cm^2 at least 15 min before UV irradiation.

Visual skin evaluation

Dermal alterations were assessed in a grading scale of 1 to 4 points for the evaluation of the test reactions, according to Glogau's reference photographic scale (Glogau, 1996), giving a visual score from 1 = no wrinkles to 4 = very marked wrinkles. An individual not involved in the treatment and irradiation work carried out the visual evaluations blind, based on group number.

Histology

Six animals per group were analyzed. Their dorsal skins were dissected using a rectangular template (2 × 2 cm) to include the entire treated areas and processed with light microscopy. Slices of 6 µm were used for the analyses and stained with hematoxylin and eosin (H&E).

Statistical Analysis

Statistical analyses were done by using the statistical package SPSS V.15.0 for Windows. Comparisons between control and treated groups were done by the Mann Whitney U test. $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Preliminary phytochemicals analysis

Qualitative phytochemical analysis conducted on *S. filiforme* using standard phytochemical screening tests, revealed the presence of polyphenols, flavonoids, anthocyanins, saponins and reducing sugars. Among these, phenolic compounds were found to be the most abundant components (72.85 ± 0.72 PE) (Table 1). Furthermore, metabolites of phenolic nature (flavonoids and anthocyanins) were detected at significant concentrations (59.09 ± 0.45 QE and 5.3 ± 0.03 ME, respectively) in this extract. Additionally, other primary metabolites were quantified and results are shown in Table 1. These results are in agreement with the major classes of metabolites found in the family *Cymodoceaceae* (Subhashini *et al*., 2013).

Table 1. Quantitative chemical composition of the crude extract of *S. filiforme*.
Tabla 1. Composición química cuantitativa del extracto crudo de *S. filiforme*

Metabolites	Concentration
Polyphenols	72.85 ± 0.72[a]
Flavonoids	59.09 ± 0.45[b]
Anthocyanins	5.3 ± 0.03[c]
Proteins	5.3 ± 0.03[d]
Carbohydrates	67.88 ± 0.54[e]
Chlorophyll a	0.25 ± 0.05[f]
Chlorophyll b	0.35 ± 0.03[f]

[a] mg pyrogallol equivalents/g dry extract (PE); [b] mg quercetin equivalents/g dry extract (QE); [c] mg malvidin-3-O-glucoside equivalents/g dry extract (ME); [d] mg BSA equivalents/g dry extract ; [e] mg D (+) galactose equivalents/g dry extract; [f] µg/mL
[a] mg equivalentes de pirogalol /g extracto seco (PE); [b] mg equivalentes de quercetina /g extracto seco (QE); [c] mg equivalentes de malvidina-3-O-glucósido /g extracto seco (ME); [d] mg equivalentes de BSA /g extracto seco; [e] mg equivalentes de D (+) galactosa /g extracto seco; [f] µg/mL

In vivo photoprotective effect of S. filiforme

The UVB exposure induced macroscopic alterations in the mouse skin within the first 48 hour such as: erythema, scabs, roughness and wrinkling. The degree of wrinkling was reduced by previous treatment with *S. filiforme*. The peak of reaction occurred between 48 and 72 h in all animals.

Figure 1 shows the degree of damage that was observed in the animals of each experimental group after seven days of treatment, according to Glogau's reference photographic scale, giving a visual score from 1=no wrinkles to 4=very marked wrinkles (Glogau, 1996). Repair of the acute damage induced by the UVB radiation with the application of the crude extract of *S. filiforme* was observed, as well as with the application of the derived aqueous fraction obtained.

In this study no evidence of sensitization on the skin of the animals in the model treated with the crude extract of *S. filiforme* was present.

The topical application of the crude extract of *S. filiforme* and the aqueous fraction, reduced the macroscopic alterations induced by UVB radiation acute exposure (Fig.1) similar to that of the positive control (Ultra Facial cream); whereas no effect was shown with chloroform fraction. The effects occurred at 240 µg.cm^{-2}. This dose was chosen because it was evaluated in previous studies with good results and also to minimize the number of experimental animals.

The skin damage was significantly suppressed by 70-80% (mean value) at the end of the application period (7 days), to the extent that there were no significant differences between the controls.

These results were corroborated by histopathology (Fig. 2), where the non treated animals showed histopathologic alterations such as acanthosis, hyperkeratosis, infiltrating inflammatory cells, dilation and growth of blood vessels and collagen fiber degradation. Nevertheless, in the groups

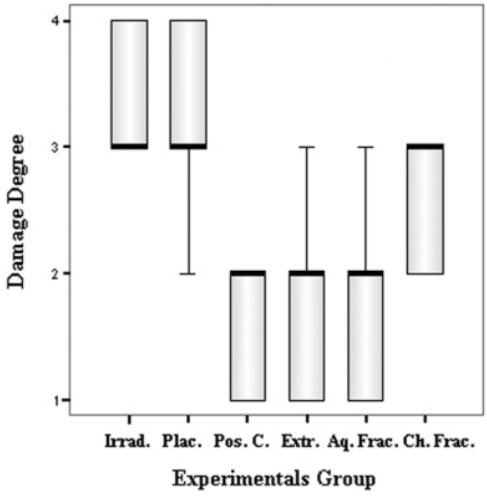

Figure. 1 Effects of the extract and fractions obtained from *S. filiforme* in the photodamaged skins with the use of UVB radiations. Irrad: Irradiated; Plac: Placebo; Pos.C: Positive Control (UF); Extr: Crude Extract of *S. filiforme*; Aq. Fr: Aqueous fraction; Ch. Fr: Chloroform fraction. Dosage: 240 µg·cm^{-2}. n=10. U de Mann Whitney test.
Figura. 1 Efectos del extracto y las fracciones obtenidas de *S. filiforme* en el fotodaño sobre la piel con el uso de las radiaciones UVB. Irrad: Irradiado; Plac: Placebo; Pos.C: Control positivo (UF); Extr: Extracto crudo de *S. filiforme*; Aq. Fr: Fracción acuosa; Ch. Fr: Fracción clorofórmica. Dosis: 240 µg.cm^{-2}. n=10. Prueba U de Mann Whitney.

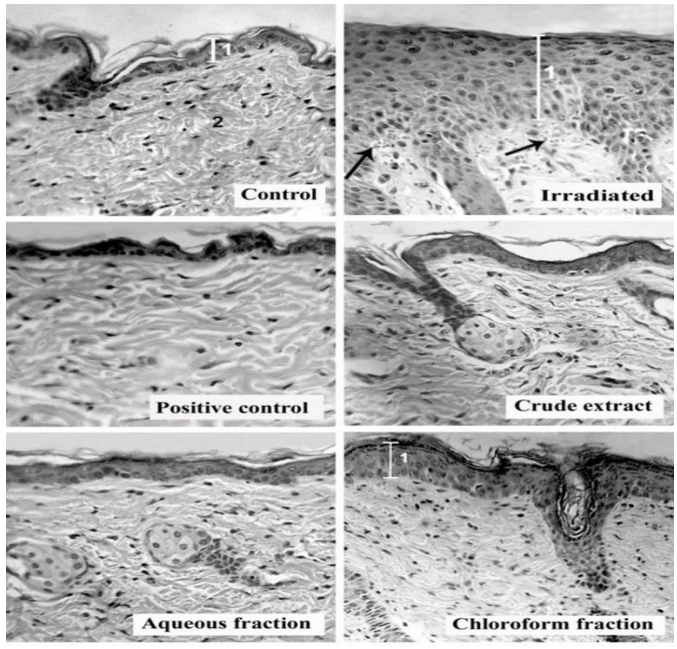

Figure 2. Histopathological study: Control: Untreated skin (1: Normal epidermis 2: Normal Dermis). Irradiated: skin UVB-photodamaged (1: marked acanthosis by epidermal hyperplasia. The arrows indicate the dilation and growth of blood vessels). Positive control*: Irradiated + topically treated with UF. Crude extract*: Irradiated + topically treated with *S. filiforme* extract. Aqueous fraction*: Irradiated + topically treated with the aqueous fraction (* the skin is comparable to Control). Chloroform fraction: Irradiated + topically treated with the Chloroform fraction (The epidermis is different to the control).
Dosage: 240 µg. cm-2. H&E stain × 400 magnifications.
Figura 2. Estudio histopatológico: Control: Piel no tratada (1: Epidermis normal 2: Dermis Normal). Irradiado: Fotodaño UVB en piel (1: Hiperplacia epidérmica marcada por acantosis. Las flechas indican la dilatación y crecimiento de los vasos sanguíneos). Control Positivo*: Irradiado + tratado tópicamente con UF. Extracto crudo *: Irradiado + tratado tópicamente con extracto de *S. filiforme*. Fracción acuosa *: Irradiado + tratada tópicamente con la fracción acuosa (* la piel es comparable al Control). Fracción clorofórmica: Irradiado + tratada tópicamente con la fracción clorofórmica (La epidermis es diferente al control).
Dosis: 240 µg. cm-2. Tinción H&E × 400 aumentos.

treated topically with the creams that contain the crude extract or aqueous fraction, the erythema was eliminated, the acanthuses and hyperkeratosis were strongly diminished, the vascular damage was reduced and a reorganization of the collagen fiber was observed, compared with the skin irradiated but not treated. This suggests recovery of the skin after treatment.

Histopathologc studies at the end of the experimental period, revealed the reorganization of parallel collagen bundles similar to that of non irradiated skin and an increase of fibroblasts. These results suggest the possibility of an increased synthesis of collagen fibers by hyperactive fibroblasts, after the application of the creams containing the crude extract that can also contribute to its skin repairing effect.

In relation to viability assessment, the extract at a dose of 1000 mg/Kg showed no cytotoxic activity in cells not exposed to UV-A as determined with the MTT assay.

The strong free radical scavenging effects of *S. filiforme* has been previosly documented (Nuissier *et al*., 2010; González García *et al*., 2011). Studies have demonstrated that the highest antioxidant contents were obtained from methanolic and aqueous-methanolic extracts of fresh leaves.

Since free radicals play an important role in UV-induced damage, the underlying protective mechanism of *S. filiforme* could be linked, either directly or indirectly, to its antioxidative capability by the scavenging of free radicals responsible for DNA damage. In addition, it is known that the crude extract of *S. filiforme* contains caffeic and ferrulic acids (Nussier *et al*., 2010), these compounds have the capacity to protect phospholipidic membranes from UV-induced peroxidation, by inhibiting propagation of the lipid peroxidative chain reaction and to react with nitrogen oxides (Saija *et al*., 1999).

CONCLUSIONS

The results of the present study indicate that *S. filiforme* is protective against chronic damage induced by UV radiation in the hairless mouse. Protection against visible changes and histological alterations were demonstrated. In recent years, there has been great interest in the use of dietary supplements that are derived from naturally occurring botanicals for the photoprotection of the skin, including protection from skin cancers. Considering these results, detrital leaves of *S. filiforme* could afford an interesting new raw material for the production of health-benefit products capable of attenuating the deleterious effects of UV on human skin. In

addition, the apparent null toxicity of the extract of *S. filiforme*, demonstrates their potential as functional, nutraceutical and pharmaceutical agent.

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