

## Photoprotective properties of a hydrophilic extract of the fern *Polypodium leucotomos* on human skin cells

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### Abstract

The effect of a hydrophilic extract of the fern *Polypodium leucotomos* (PLE) has been investigated in terms of photoprotection against UV-induced cell damage. PLE efficiently preserved human fibroblast survival and restored their proliferative capability when the cells were exposed to UVA light. This effect was specific and dose-dependent. Photoprotection was not restricted to fibroblasts, as demonstrated by its effect on survival and proliferation of the human keratinocyte cell line HaCat. Finally, treatment of the cells with PLE prevented UV-induced morphological changes in human fibroblasts, namely disorganisation of F-actin-based cytoskeletal structures, coalescence of the tubulin cytoskeleton and mislocalization of adhesion molecules such as cadherins and integrins. Our *in vitro* results demonstrate the photoprotective effect of PLE on human cells and support its use in the preventive treatment of sunburning and skin pathologies associated with UV-mediated damage.

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**Keywords:** *P. leucotomos*; Ultraviolet; Photoprotection; Cytoskeleton; Fibroblast; Keratinocyte

### 1. Introduction

The effect of overexposure to ultraviolet radiation in the development of skin diseases and melanoma has been clearly demonstrated in recent years [1,2], and thus the need for developing protective reagents is an active field of study. Protection should prevent the development of severe burns, hyperpigmentation, photoallergy and phototoxicity, skin cancer and chronic skin damage, and premature aging. There has been considerable controversy on the type of UV radiation involved in the injurious effects described above. Although it was first conceived that UVB was most involved in these processes, the general consensus points to a role of both UVA and UVB in the development of skin cancer [3] and melanoma [4].

It is well stated that any live organism exposed to UV

radiation reacts in different ways, such as avoidance of the UV source (phototaxis), screening under inert materials, and specially the production of photoprotective compounds that specifically screen UV radiation, such as mycosporine-like amino acids, scytonemin secreted by cyanobacteria, flavonoids secreted by plants and melanin expressed by skin cells in animals and humans [5]. Thus, agents that may induce skin damage in animals may also induce the production of secondary metabolites entitled with photoprotective effect in organisms such as microorganisms or higher plants [5].

The beneficial effect of an extract of the fern *Polypodium leucotomos* (PLE), minimizing photoaging changes and preventing acute sunburn, has been described [6,7]. PLE has also been reported to inhibit the formation of reactive oxygen species induced by UV light [8] and to possess antiinflammatory properties [9]. In this report, we describe the *in vitro* photoprotective effect of the PLE, as it preserves the proliferative ability of UV-treated cells and protects the cells from UVA-induced cytoskeletal changes. We propose that the PLE exhibits photoprotective prop-

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erties in vitro, as determined by reduction of the deleterious effect of UVA light on both cellular proliferation and morphology.

## 2. Materials and methods

### 2.1. Cells and reagents

Fibroblasts were obtained from clinical samples of healthy volunteers. The keratinocyte cell line HaCat was obtained from the American Tissue Culture Collection (ATCC). The following reference antioxidants, all of them from Sigma (St. Louis, MO, USA), were employed: *N*-acetylcysteine (NAC); trolox (a synthetic analog of  $\alpha$ -tocopherol); and ascorbic acid. The doses employed in this study (see Table 1) have been described in the literature in assays similar to those reported herein [10–13]. The mAb TS2/16 ( $\beta$ 1 integrin chain), HUTS-21 (activated  $\beta$ 1 chain), Lia1/1 (tetraspanin CD151) and Chelo-3/2 (vimentin, an intermediate filament) have been described previously [14,15]. Commercial mAb against  $\alpha$ -tubulin and pan-cadherin were from Sigma and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Hydrophilic extract of PLE was prepared according to the following protocol: dry *P. leucotomos* leaves were extracted employing water as solvent at 55 °C, allowing recirculation of the solvent until a plateau of antioxidant activity (determined by FRAP [16]) and phenolics concentration (determined by the Folin–Ciocalteu method

[17]) were reached, parameters usually achieved after 4 h of processing. Finally, the extract was vacuum-concentrated and kept at 4 °C and light-shielded until use.

### 2.2. Irradiation

The ultraviolet irradiation systems used in this study consisted of a 40 W UVA-emitting fluorescent tube (320–400 nm) made by Sylvania (F490T12BL/HO) or a 40 W Westinghouse (FS40) UVB-emitting lamp. The flux of irradiance of the emitted light was measured by an IL-1400A radiometer unit equipped with two separately calibrated UVA- and UVB-detecting probes. Exposure doses of UVA were delivered in J/cm<sup>2</sup> (usually 1 J/cm<sup>2</sup>), and UVB doses were delivered in mJ/cm<sup>2</sup>. Fibroblast monolayers were washed twice and irradiated in the presence of PLE or control compound at different doses determined in each experiment. Fresh medium was then added and the cells were incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere until the samples were analyzed.

### 2.3. MTT assay

Cell survival was estimated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT (Sigma), to a colored formazan product. Cells were treated as indicated and MTT (0.5 mg/ml in phosphate-buffered saline, PBS) was added to each well and incubated for 2 h at 37 °C. The medium was then carefully aspirated, and 200  $\mu$ l of acidified isopropanol was added to solubilize the colored formazan product. Absorbance was read at 550 nm on a scanning multiwell spectrophotometer (Bio-Rad) after vigorous shaking of the plates for 5 min.

### 2.4. Proliferation assay

Fibroblasts or HaCat cells were seeded in 24-well tissue plates under subconfluent conditions and were irradiated in the presence or not of the different treatments indicated in each case. Growing medium was substituted by fresh OptiMem medium supplemented with 0.5% fetal calf serum (FCS), and <sup>3</sup>H-thymidine (1  $\mu$ Ci/ml) was added. Then, cells were incubated at 37 °C for 24 h, washed twice with ice-chilled PBS and fixed for 20 min at 4 °C with chilled 10% trichloroacetic acid (TCA). TCA was removed and the wells were washed twice with cold ethanol, air-dried and the cells were solubilized with 200  $\mu$ l 0.4 M NaOH at 65 °C for 10 min. After plate cooling, 5  $\mu$ l acetic acid was added to each well and the content was transferred to scintillation vials in which it was mixed with 3 ml scintillation fluid. Finally, radioactivity in each vial was measured for 1 min in a  $\beta$  radiation counter.

### 2.5. Indirect immunofluorescence

Human fibroblasts were cultured on glass coverslips,

Table 1  
Effect of PLE and different antioxidants on the survival of 'in vitro' UVA-irradiated human fibroblasts

Compound	Dose	Survival (%)
Untreated	–	29 $\pm$ 3
PLE	0.1 mg/ml	53 $\pm$ 1
	0.5 mg/ml	61 $\pm$ 2
	1 mg/ml	87 $\pm$ 2
<i>N</i> -Acetylcysteine	0.005 M	52 $\pm$ 8
	0.01 M	62 $\pm$ 11
Trolox	6.25 $\mu$ g/ml	30 $\pm$ 7
	12.5 $\mu$ g/ml	29 $\pm$ 5
	25 $\mu$ g/ml	33 $\pm$ 6
	50 $\mu$ g/ml	50 $\pm$ 1
Ascorbic acid	10 <sup>-6</sup> M	35 $\pm$ 5
	10 <sup>-4</sup> M	41 $\pm$ 8
	2 $\times$ 10 <sup>-2</sup> M	72 $\pm$ 12

Survival was determined by the MTT method as described in Materials and methods. Cells were treated with the indicated compounds at doses indicated and subsequently irradiated with 1 J/cm<sup>2</sup> UVA light. Data correspond to the mean $\pm$ S.D. of three independent experiments performed in triplicate.

irradiated in either the presence or absence of PLE extract and fixed with 4% formaldehyde for 10 min at room temperature. For intracellular staining, the cells were permeabilized with 0.5% Triton X-100 for 5 min at room temperature. The cells were incubated with the indicated murine mAb for 30 min at 37 °C, washed twice in Tris-buffered saline (TBS) and incubated with secondary antibodies coupled to the fluorescent dye Alexa-488 (Molecular Probes, Eugene, OR, USA) or with Alexa-568-phalloidin (Molecular Probes) for actin staining. Samples were then mounted on glass slides, glued with Mowiol (Calbiochem, La Jolla, CA, USA) and examined with a Leica DMR photomicroscope (Leica, Mannheim, Germany) with oil immersion objectives (63× was employed for all images). Finally, images were processed in a Leica Q550CW workstation (Leica Imaging Systems, Cambridge, UK), equipped with Leica QFISH software V1.01.

### 3. Results

#### 3.1. Treatment with an extract derived from *P. leucotomos* preserves the proliferative capability of irradiated human fibroblasts

To assess the photoprotective capability of PLE in vitro, human fibroblasts from healthy donors were irradiated with 1 J/cm<sup>2</sup> UVA light in the presence or absence of PLE and survival was measured by MTT as described in Materials and methods. As control, cells were incubated with different doses of other antioxidants such as *N*-acetylcysteine [18], trolox or ascorbic acid (Table 1). PLE induced a significant increase in the level of fibroblast survival that was dose-dependent (Table 1). Control antioxidants, although preserving survival above the untreated controls, were not as efficient as 1 mg/ml PLE in enhancing cell survival (Table 1). These data were extended to UVA-treated fibroblast growth through proliferation assays. As shown in Fig. 1A, PLE-treated, UVA-irradiated cells exhibited a partial capability to proliferate when compared to untreated or NAC-treated cells. PLE had a negligible effect on cell proliferation by itself (data not shown). In addition, the protective effect of PLE on cell proliferation was found to be dose-dependent (Fig. 1B).

To determine the extent of the photoprotective effect of PLE, the cells were incubated with 1 mg/ml PLE and irradiated with different doses of UVA light. Fig. 2 shows that complete photoprotection is achieved up to 1 J/cm<sup>2</sup>, declining thereafter, although significant protection is observed up to 5 J/cm<sup>2</sup>. On the other hand, purified low-molecular-weight saccharides of PLE exerted no effect under the same conditions (data not shown). These results indicate that PLE exerts a UV dose-dependent photoprotective effect on human cells that could be extended to longer periods of time.

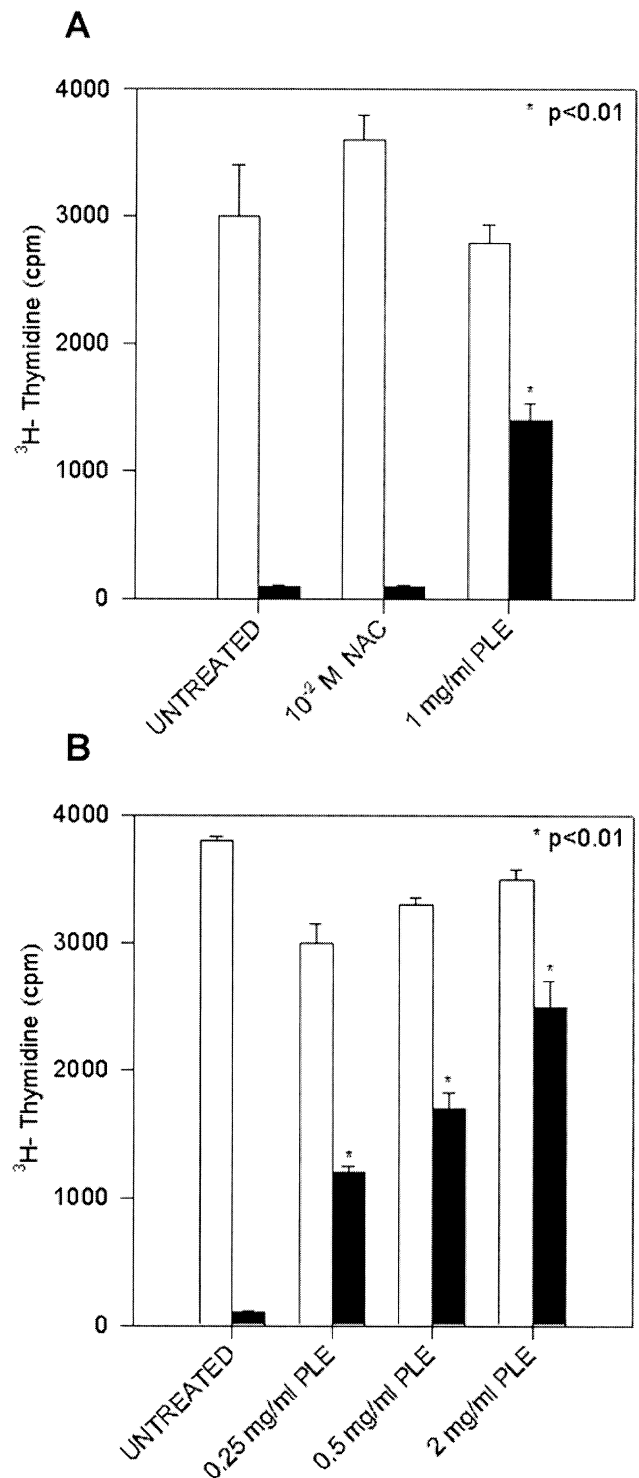


Fig. 1. Effect of PLE on the proliferation of irradiated human fibroblasts. Human fibroblasts were treated with 0.01 M *N*-acetylcysteine, 1 mg/ml PLE or vehicle alone (A) or with the indicated doses of PLE (B) and then irradiated with 1 J/cm<sup>2</sup> of UVA light (black bars) or left untreated (white bars). Proliferation experiments were carried out as indicated in Materials and methods. Results represent the mean ± S.D. of three experiments performed in triplicate. The significance of the observations was determined with Student's *t*-test.

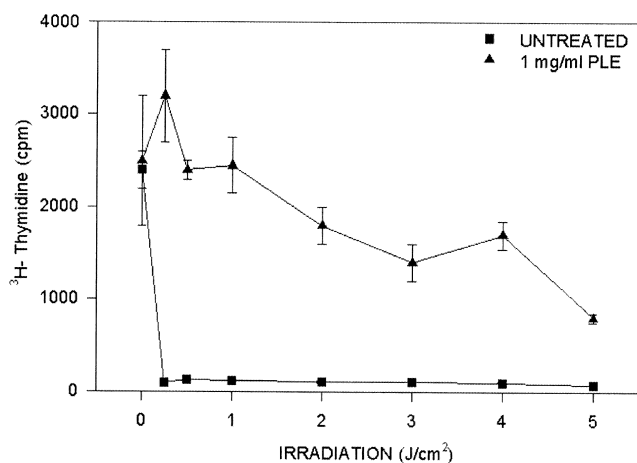


Fig. 2. Protective effect of PLE against UVA-induced damage on human fibroblast proliferation. Human fibroblasts were treated with 1 mg/ml PLE or vehicle alone and then irradiated with the indicated doses of UVA light. Proliferation experiments were carried out as indicated in Materials and methods. Results represent the mean  $\pm$  S.D. of three experiments performed in triplicate.

### 3.2. Treatment with PLE preserves the proliferative capability of irradiated human keratinocytes

To investigate whether the effect of PLE could also be extended to keratinocytes, which are the cells mainly exposed to UV light, a human keratinocyte cell line, HaCat, was employed in irradiation assays. First, the photoprotective effect of PLE was assessed in keratinocyte survival. PLE-treated cells were irradiated with both UVA and UVB light. Due to the high resistance of these cells to irradiation, doses of 10 and 15 J/cm<sup>2</sup> UVA light were employed (Table 2 and Fig. 3). Separate samples were also irradiated with 10 mJ/cm<sup>2</sup> UVB light (see below). PLE partly preserved HaCat survival in response to both UVA (Table 2) and UVB (30  $\pm$  3% survival of untreated cells vs. 52  $\pm$  8 and 75  $\pm$  6% of cells treated with 0.1 and 1 mg/ml PLE, respectively) as determined by MTT. To ascertain the effect of PLE on cell proliferation, cells were treated with 1 mg/ml PLE and subjected to irradiation with UVA light as described before. 1 mg/ml PLE also exerted a protective effect on cell proliferation, although to a lower extent compared with normal fibroblasts (Fig. 3), which

Table 2  
Effect of PLE on cell survival of in vitro UVA-irradiated human HaCat cells

Compound	Survival (%) UVA
Untreated	40 $\pm$ 5
PLE (0.1 mg/ml)	64 $\pm$ 6
PLE (1 mg/ml)	90 $\pm$ 8

Survival was determined by the MTT method as described in Materials and methods. Cells were treated with the indicated compounds and subsequently irradiated with 10 J/cm<sup>2</sup> UVA light. Data correspond to the mean  $\pm$  S.D. of three independent experiments performed in triplicate.

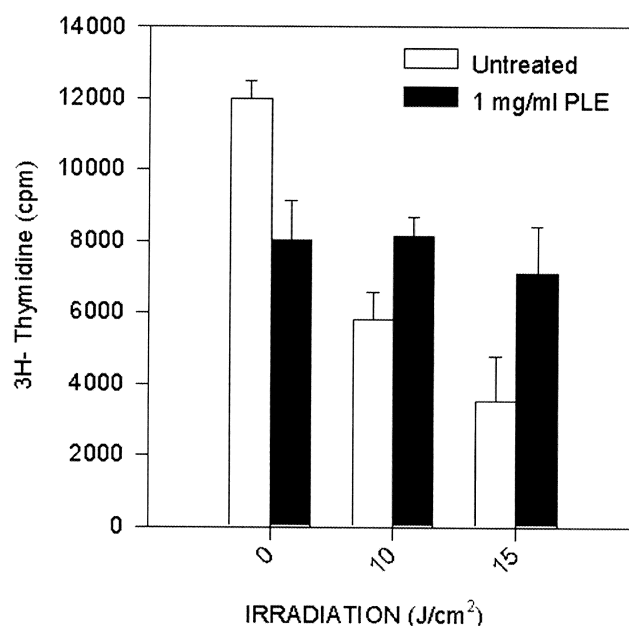


Fig. 3. Effect of PLE in proliferation of irradiated human keratinocytes. Human keratinocytes of the HaCat line were treated with 1 mg/ml PLE or vehicle alone, then irradiated with the indicated doses of UVA light as indicated. Proliferation experiments were carried out as indicated in Materials and methods. Results represent the mean  $\pm$  S.D. of three experiments performed in triplicate. The significance of the observations was determined with Student's *t*-test.

may result from differences in both the cell type or the fact that the fibroblasts employed are freshly isolated primary cells, whereas HaCaT are transformed cells.

### 3.3. Effect of PLE on cytoskeleton organisation and subcellular localisation of adhesion molecules of human fibroblasts

UVA irradiation causes dramatic effects on the morphology of the cells [19,20]. This extent has also been investigated by indirect immunofluorescence. First, we established that PLE by itself did not cause any appreciable change in the morphology of the cells (data not shown). As shown in Fig. 4A, exposure of human fibroblasts to 1 J/cm<sup>2</sup> UVA light induced changes in the shape of the cells and their cytoskeletal structures. Definite cell–cell contacts were lacking, and F-actin staining revealed that straight, parallel stress fibers were disorganised by UVA treatment, leaving only unaligned F-actin bundles along the periphery of the cell. Most interestingly, treatment of the cells with 1 mg/ml PLE blocked UVA-induced F-actin changes and allowed the cells to retain their stress fibers. Changes were also evident in the tubulin cytoskeleton. Although the perinuclear position of the centrosome was not affected, the microtubule network lost coalescence and the individual microtubules were more evident. PLE also prevented this effect of UVA light. On

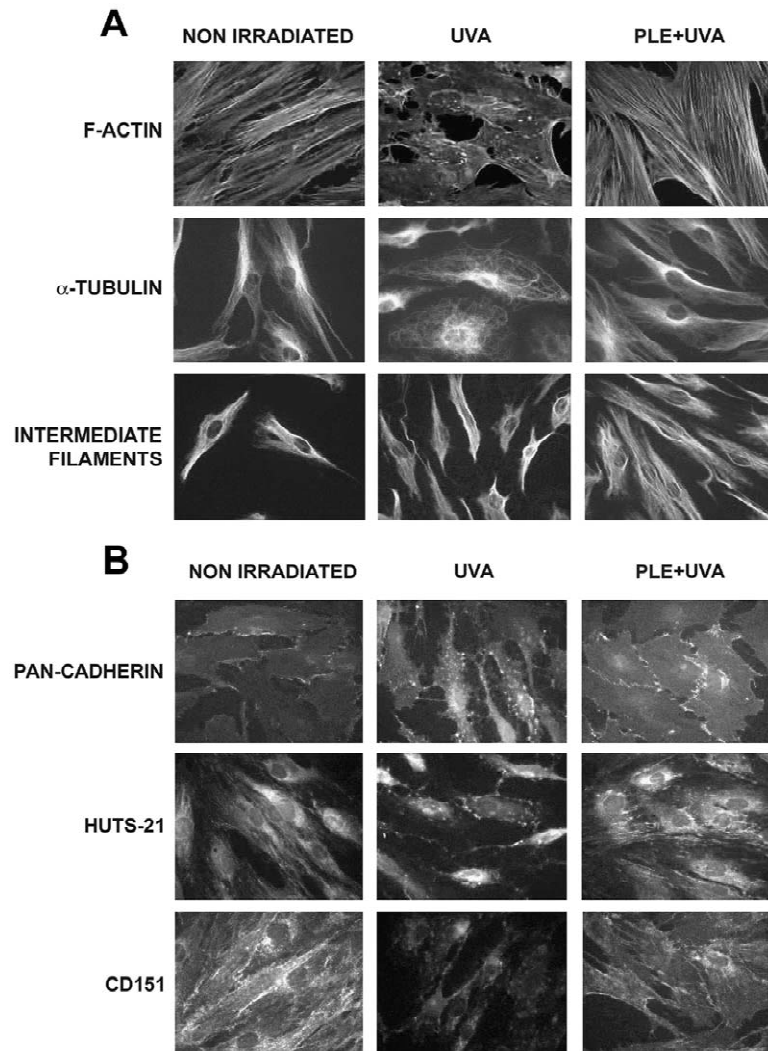


Fig. 4. Effect of PLE on the cytoskeleton organization and adhesion molecule localization in human fibroblasts. Human fibroblasts were seeded in subconfluent conditions, treated or not with 1 mg/ml PLE, irradiated with 1 J/cm<sup>2</sup> UVA light as indicated, fixed and cytoskeletal structures (A) and adhesion molecules (B) stained with specific antibodies or fluorescence-conjugated phalloidin.

the other hand, the effect on the intermediate filament network was not so obvious, irradiated cells exhibiting similar immunofluorescence distribution pattern to that of non irradiated cells or PLE-treated UVA-irradiated cells.

Changes were also observed in adhesion molecule distribution patterns. E-cadherin staining is normally confined to intercellular contacts, as shown in Fig. 4B (non irradiated panel). Upon UVA treatment, dense patches of E-cadherin can be observed. PLE treatment restored cadherin localisation at intercellular cell–cell contacts. On the other hand, the activated  $\beta$ 1 integrin chain, detected by the antibody HUTS-21 [14], showed a punctate pattern along the cell body consistent with focal adhesions structures, and UVA treatment induced a decrease in  $\beta$ 1 clustering along the cell body that was preserved in the cells treated with PLE. Finally, staining of the integrin-associated tetraspanin molecule CD151/PETA-3, a marker of cell-to-cell contacts [21], showed that UVA-treatment

induced the downregulation of this molecule, which was reversed by treatment of the cells with PLE.

#### 4. Discussion

In this report, we have demonstrated the ability of an extract of the fern *P. leucotomos* to prevent UV-related damage at a cellular level in human cells.

We have first evaluated the screening capability of the PLE. Absorbance data demonstrated that the PLE absorbed a negligible amount of UVA light (data not shown), thus suggesting that the observed effect on cell cultures was not due to an actual reduction of the exposure of the cells to the UV light caused by its partial absorption by PLE.

Proliferation has been chosen as a marker of cell viability for several reasons, the main one being that UV light has been demonstrated to damage the cytoskeleton of

the cell, which is clearly necessary for proliferation due to its role in the formation of the mitotic spindle and cytokinesis. As shown herein, PLE was able to protect human fibroblasts from UVA-induced damage, increasing the levels of proliferation well above vehicle-treated cells. This result concurs with other reports in which the extract demonstrated clinical efficiency in the regeneration and treatment of the skin during chronic diseases such as psoriasis or atopic dermatitis [6,7]. The effect was demonstrated to be specific due to its dose-dependence, and it was established that the moiety responsible of the photoprotective effect was not sugar-based, as glycidic fractions of the extract did not retain the beneficial effect.

Photoprotection was firmly established in two different human cell models, keratinocytes and fibroblasts, what suggests that the specificity of the product is not limited by cell lineages, and suggests its employment for tissue regeneration. On the other hand, it has been established that UV light or endotoxin induce the synthesis and release of TNF- $\alpha$  by human keratinocytes [22]. These facts, together with its ability to inhibit TNF- $\alpha$  induction upon LPS treatment [9], suggests that PLE presents a dual profile in the treatment of skin lesions, protecting the tissue from UV-induced damage and limiting the extent of the inflammatory response upon damage, making it a promising candidate in the treatment of UV-damage related skin diseases that cause a depletion of Langerhans cells, which has been related to TNF- $\alpha$  accumulation [23].

The finding that PLE blocks UV-induced cytoskeletal rearrangements suggests that its photoprotective effect may not be limited to DNA synthesis, but also to cytoskeletal mechanisms. In this regard, UV damage has been demonstrated to induce inhibition of actin-based stress fibers, in a process similar to that induced by serum depletion, that can be reversed by LPA addition to the medium [24]. This effect has been demonstrated to depend on the activity of the small GTPase Rho, but if UV damage induces a down-regulation of RhoA activity remains to be demonstrated. However, the dual role of RhoA in both actin and tubulin reorganization [25], and the effect of UV damage on both cytoskeletal structures makes it a promising candidate for mediating the effect of the extract, further supported by its protective effect on other RhoA-dependent structures such as focal adhesions enriched in activated  $\beta$ 1 integrins, as detected by the HUTS-21 antibody [14], or cadherin-rich cell–cell contacts [26].

In summary, the evidences presented herein support the photoprotective role of PLE and suggest a possible mechanism for its beneficial effect on the treatment of UV-induced skin damage.

## 5. Nomenclature

PLE	<i>Polypodium leucotomos</i> extract
NAC	<i>N</i> -Acetylcysteine

FRAP	Ferric-reducing ability of plasma
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TCA	Trichloroacetic acid
TNF	Tumor necrosis factor
LPA	Lysophosphatidic acid

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