
Oral *Polypodium leucotomos* extract decreases ultraviolet-induced damage of human skin

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Background: UV radiation induces damage to human skin. Protection of skin by an oral photoprotective agent would have substantial benefits.

Objective: We investigated the photoprotective effect of oral administration of an extract of the natural antioxidant *Polypodium leucotomos* (PL).

Methods: A total of 9 healthy participants of skin types II to III were exposed to varying doses of artificial UV radiation without and after oral administration of PL (7.5 mg/kg). At 24 hours after exposure the erythema reaction was assessed and paired biopsy specimens were obtained from PL-treated and untreated skin.

Results: A significant decrease in erythema was found in PL-treated skin ($P < .01$). Histologically, PL-treated biopsy specimens showed less sunburn cells ($P < .05$), cyclobutane pyrimidine dimers ($P < .001$), proliferating epidermal cells ($P < .001$), and dermal mast cell infiltration ($P < .05$). A trend toward Langerhans cell preservation was seen.

Conclusion: Oral administration of PL is an effective systemic chemoprotective agent leading to significant protection of skin against UV radiation. (J Am Acad Dermatol 2004;51:910-8.)

Exposure of human skin to sunlight, containing UV radiation (UVR) A and B, leads to deleterious effects on skin such as sunburn, immune suppression, pigmentary changes, photoaging, and

Abbreviations used:

CPD: cyclobutane pyrimidine dimer
MED: minimal erythema dose
PL: *Polypodium leucotomos*
UVR: ultraviolet radiation

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skin cancer.¹ The mechanism of such cutaneous damage induction is complex, but can be broadly divided in direct oxygen-independent damage through absorption of photons, and in oxidative damage, caused by formation of free radicals and reactive oxygen species.² This is why antioxidants have been increasingly studied as inhibitors or quenchers of UV-induced cutaneous damage. Currently the most widely used method of protection against UV-induced damage is the use of topical sunscreens enriched with UV-absorbing chemicals. A systemic photoprotective agent would obviously have an advantage over topical protection as this would provide uniform, total body surface protection without the variance in protection commonly observed with topical sunscreens.³ Attempts have been made to investigate the photoprotective effects

of systemic antioxidants. However, oral antioxidants such as tocopherol, ascorbate, or carotenoids have shown varying effects as some studies show a slight to moderate increase in the minimal erythema dose (MED) in human beings,⁴⁻⁹ whereas others did not find an increased MED,¹⁰⁻¹² reduction of sunburn cells,^{10,13} or protection against DNA damage.¹¹ When a photoprotective effect was observed, this was usually after prolonged administration of the antioxidants.^{4,5,8} Green tea also seems a promising antioxidant, but its protective effect after oral administration has so far not been evaluated in controlled human studies.¹⁴

In this study we investigated the photoprotective effect of orally administered *Polypodium leucotomos* (PL) against acute UV-induced damage to human skin using a solar simulator. PL is an extract from a fern plant grown in Central America.¹⁵ In vitro studies have shown that PL acts as an effective antioxidant by quenching superoxide anion, singlet oxygen, lipid peroxides, and the hydroxyl radical.^{16,17} Previous studies showed that topical and oral PL decreased acute sunburn response and resulted in Langerhans cell preservation of human skin when exposed to sunlight¹⁸ and psoralen-UVA.¹⁹ The goal of this study was to further analyze the ability of orally administered PL to decrease UV-induced erythema and the resulting histologic skin damage under carefully controlled laboratory conditions.

METHODS

Participant selection

We included 9 healthy participants with skin phototype II or III in this open-label study after they read and signed a written informed consent form and protocol approved by an institutional review board of Massachusetts General Hospital, Boston, Mass. The study was conducted during a period of 1 year in our laboratories. The group consisted of 5 men and 4 women with ages ranging from 25 to 46 years. Participants were excluded when they had a personal or family history of skin cancer, had a history of abnormal photosensitivity, or were taking any drug that might alter the response of skin to UVR. We used the skin of the back of each participant for these studies, which had to be free of any blemishes and not exposed to sunlight or artificial UVR sources (tanning booth) for at least 8 weeks before the study.

Radiation source and exposure conditions

A solar simulator consisting of a 1000-W high-pressure xenon arc lamp (Oriol Corp, Stratford, Conn) emitting a collimated beam and equipped with a 2-mm filter (WG-305, Schott Glas, Mainz,

Germany) and a first surface mirror (Edmund Scientific, Barrington, NJ) was used as radiation source (305-400 nm). A high-velocity fan eliminated any impact of heat from the infrared radiation of the lamp on the exposed skin of the participant. The emitted UVR intensity of the source was measured with a calibrated radiometer (International Light, Newburyport, Mass) before each experiment when the lamp had warmed up for 30 minutes, and after each experimental protocol to ensure stability of the UVR output, which was usually around 0.2 mW/cm². The skin was irradiated through adhesive UVR-reflecting aluminum stickers containing 6 or 7 exposure windows, each 3.3 cm² in size (DV Die Cutting Inc, Danvers, Mass). One phototest consisted of exposure of each skin site to UVR doses with a relative increase of 6% to 33% to obtain an exposure range with regular incremental UVR doses. A dose between 2 to 3 times the MED was always included when a biopsy specimen was obtained. The MED was defined as the minimal dose of UVR inducing confluent erythema at 20 to 24 hours with 4 sharp borders of the exposed skin site. From the 7 exposed skin sites, either the first, second, third, or fourth site was exposed to the MED dose, and either the fifth or seventh site was exposed to the 2× to 3× MED. During exposures the rest of the skin of the back of the participant was covered by an opaque UV-protective cloth. The distance between the exposed skin and the solar simulator was kept constant by stabilizing the back skin against an aluminum template located at a fixed distance from the exit port of the lamp.

Study design

Before the start of the study each participant's MED was assessed. Once the MED was known, a set of 6 or 7 skin sites was exposed to UVR without PL. The erythema reaction of all participants was visually graded 24 hours later. A 3-mm punch biopsy specimen was taken from 5 participants after 24 hours and from two participants 72 hours after exposure.

After completion of this initial part of the protocol, the participants received the first dose of oral PL the evening before the second exposure. The next day each participant received the second dose of oral PL, after which they were exposed to the same set of fluences given in the first part of the protocol, but at 5 different time points, ie, after 30 minutes, 1 hour, 1 hour and 30 minutes, 2 hours, and 3 hours of ingestion of oral PL. Skin erythema was again evaluated after 24 hours, and a second skin biopsy specimen was taken from the same participants: 5 after 24 hours, and two after 72 hours. Specifics

about the biopsy specimens are given in the "Histology" section.

The capsules containing PL (180 mg each; Fernblock, Industrial Farmaceutica Cantabria, SA, Madrid, Spain) were administered orally in a dose of 7.5 mg/kg body weight.

Clinical evaluation

Clinical evaluation of the erythema response of every exposed skin site was performed by at least two experienced investigators using a scoring system for the intensity of erythema and edema ranging from grades 0 to 4 (0 = no erythema; 1 = trace erythema; 2 = visible, not confluent erythema, no sharp borders; 3 = confluent erythema with 4 sharp borders and no edema (MED); 4 = intense erythema with edema). The mean of the grades of each skin site was calculated for all participants at every time point of exposure.

Histology

Biopsy specimens taken during the first part of the protocol were obtained from the skin site exposed to the 2× to 3× MED. Likewise, the biopsy specimen obtained in the second part of the protocol was taken from the skin site exposed to the same fluence, from the phototest at the time point showing maximal photoprotection. All skin specimens were cut in half. One half was fixed in 10% buffered formalin and embedded in paraffin for sectioning and microscopic evaluation. Sections measuring 3 μm were routinely stained with hematoxylin and eosin for histologic gross evaluation and quantitative assessment of sunburn cells. The other half was imbedded in OCT compound (Miles Inc Diagnostic Division, Elkhart, Ind) and stored at -70°C.

Immunohistochemistry

Sections measuring 5 μm were used for immunohistochemical analysis. They were deparaffinized with xylene and rehydrated with graded ethanols. Endogenous peroxidase was blocked with hydrogen peroxide, and the samples were then rinsed in phosphate-buffered saline. To achieve adequate intensity signals with the respective antibodies, heat-induced antigen retrieval was carried out by microwave pretreatment in citric acid buffer (10 mmol/L; pH 6.0) for 20 minutes. For reduction of background labeling, the sections were blocked for 30 minutes in normal horse serum. The sections were later incubated overnight at 4°C with commercially available antibodies: (1) prediluted anti-CD1a for Langerhans cells (clone O10, catalog No. 1590) (Immunotech, Marseille, France)²⁰; (2) 1:150 diluted anti-Ki67 for proliferating keratinocytes (clone MIB-

1, catalog No. M72470) (Dako, Glostrup, Denmark)²¹; (3) 1:100 diluted antitryptase for mast cells (clone AA1, catalog No. M7057) (Dako)²²; and (4) 1:10 diluted anti-CD31 for endothelial cells (clone JC/70A, catalog No. M0823) (Dako).²³ After washing with phosphate-buffered saline, sections were incubated with biotinylated secondary antibody, avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories, Burlingame, Calif), and then 3,3'-diaminobenzidine. Each section was counterstained with hematoxylin, dehydrated, and covered with a coverslip.

For detection of UV-induced cyclobutane pyrimidine dimer (CPD) we fixed 5-μm sections of frozen tissue (from 3 participants 24 hours after UVR) on slides with ice-cold methanol-acetone (1:1) for 10 minutes at -20°C then air-dried. For immunohistologic detection of CPDs, we incubated acetone-fixed sections with CPD-specific antibodies overnight, then used a mouse tissue detection system (AEC, LabVision, Fremont, Calif) as described in the manufacturer's protocol. Positive and negative controls were always included in each staining run.

Quantitative histologic analysis

All parameters listed below, except for CPDs, were quantified in a blinded fashion.

Sunburn cells

Sunburn cells were defined as cells with a hyper-eosinophilic cytoplasm and a dense, small, dark, and irregularly formed nucleus in comparison with neighboring cells, and located in the epidermis away from areas of blistering or crush artifacts.²⁴ Sunburn cells were quantified in 4 entire sections per specimen (10-11 fields/section) using a light microscope at 40 times original magnification. Using an eyepiece micrometer, the average number of sunburn cells per millimeter of epidermal length was calculated.

CPDs

The number of CPD-positive cells was counted in approximately 5 fields of view in 6 paired biopsy specimens at 20 times original magnification. CPD-positive cells were defined as bright red—stained cells. The number of CPD-positive cells was calculated per millimeter epidermis.

Proliferating cells

Ki67 immunoreactivity (proliferating cells) was quantified with an image analysis system (Visilog, Noesis, France). The equipment used included a microscope (Elipse E400, Nikon, Tokyo, Japan) with a 20× objective lens, a digital color camera (Polaroid Corp, Waltham, Mass), and image-processing and

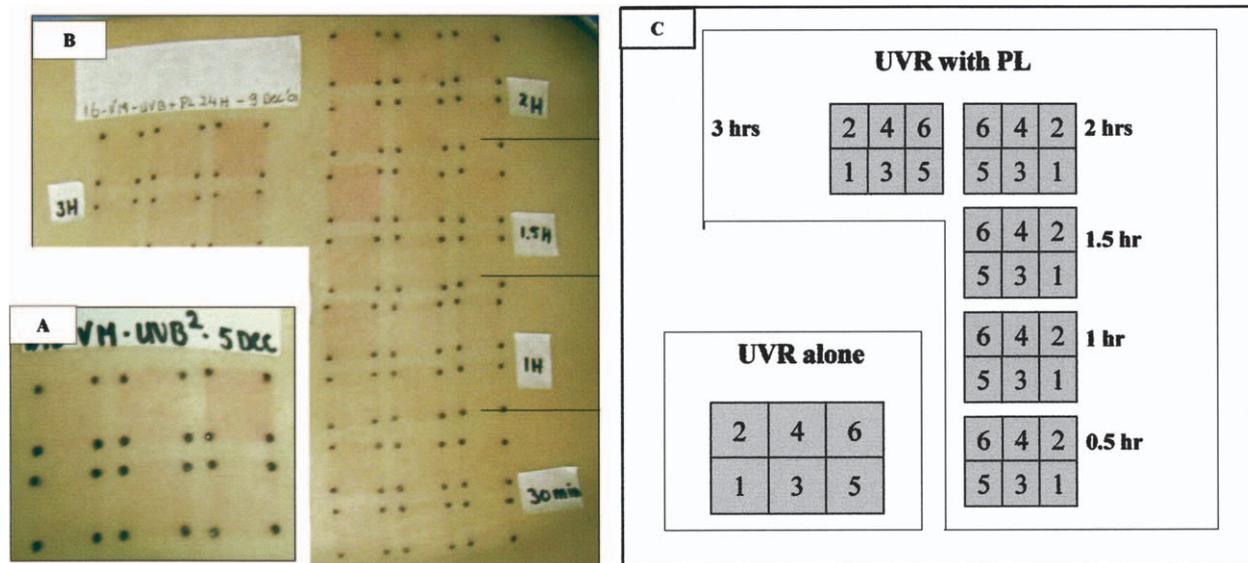


Fig 1. Pictures of one participant. Erythema reaction 24 hours (hrs) after exposure to UV radiation (UVR) alone (A) and with *Polypodium leucotomos* (PL). B, Schematic illustration of UVR intensities of each skin site shown in pictures, indicated by numbers in squares (1 = lowest UVR intensity, 6 = highest UVR intensity). C, Some sites are mirrored.

analysis software (Version 5.2, Visilog). This program is able to differentiate and count hematoxylin-stained objects and Lag-red/brown (3,3'-diaminobenzidine-stained) objects. The percentage of proliferating epidermal cells was calculated by dividing the number of immunoreactive nuclei by the total number of epidermal nuclei.

Langerhans cells and mast cells

CD1a⁺ cells (Langerhans cells) and tryptase-positive cells (mast cells) were quantified by light microscopy using a 40× objective lens and an eyepiece micrometer. A cell with a nucleus and clear immunoreactivity was considered a positive cell. In each biopsy specimen the number of positive cells was counted in 8 to 10 consecutive fields, and then calculated per square millimeter of epidermal or papillary dermal surface, respectively. To determine the number of mast cells, the micrometer and grid were aligned on one edge along the epidermal dermal junction.

Microvessels

Using a 40× objective lens, 8 to 10 consecutive fields were counted. CD31⁺ cell (endothelial cell) clusters consisting of more than two cells and large microvessels were included in the microvessel count.²⁵ The number of microvessels per square millimeter dermis was counted, and vasodilatation assessed as a percentage of the total surface area

occupied by vessels divided by the total dermal surface area.

Statistical analysis

Clinically scored erythema grades were analyzed using analysis of variance test. Mann-Whitney tests were performed to compare the number of sunburn cells, proliferating cells, and microvessels and vasodilation in PL-treated and PL-untreated skin specimens. Repeated measurement multivariate analysis of variance test was performed to compare the number of CPD-positive cells, Langerhans cells, and mast cells. Analyses were performed on data obtained either at 24 hours, 72 hours, or combined paired data. A *P* value of .05 or smaller was considered statistically significant.

RESULTS

Clinical results

PL-treated skin showed clearly less acute erythema reaction compared with PL-untreated skin when evaluated at 24 hours after exposure (Fig 1). The difference in erythema was quantified by comparing the means of the erythema grading between PL-treated and PL-untreated skin (Fig 2). The difference between the means of erythema grading of PL-treated and PL-untreated skin was statistically significant (*P* < .01) up to 2 hours after administration of PL. After 3 hours of PL administration, there was no significant difference between the

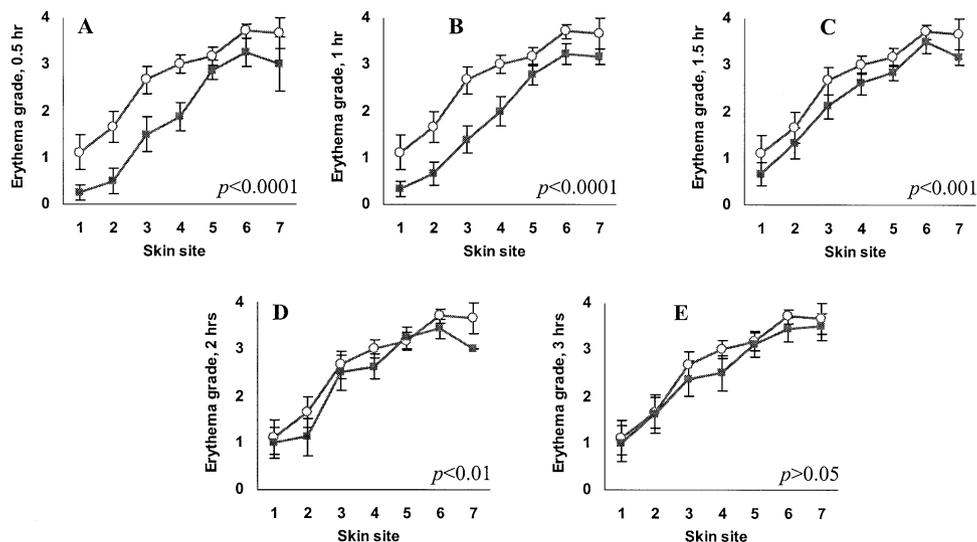


Fig 2. Open circles represent mean grades of skin sites exposed to UV radiation (UVR) without *Polypodium leucotomos* (PL), and closed squares to UVR with PL after: 30 minutes (A); 1 hour (B); 1 hour and 30 minutes (C); 2 hours (D); and 3 hours (E). Values are mean of erythema grades at 24 hours (y-axis) of each exposed skin site (x-axis) \pm SEM. PL-treated skin has statistically significant lower erythema grades up until 2 hours after administration (N = 9 participants).

means of erythema of PL-treated and untreated skin ($P > .05$).

Histologic results

Hematoxylin and eosin—stained sections from PL-treated skin clearly showed gross morphologic differences when compared with PL-untreated skin at 24 hours. PL-treated skin showed less UV-induced epidermal damage such as maturation disarray, microvesiculation, and vacuolization of keratinocytes, whereas this was more noticeable in PL-untreated skin (Fig 3, A). The number of sunburn cells per millimeter of epidermis was significantly lower in PL-treated skin when compared with PL-untreated skin at 24 hours ($P = .03$) (Fig 3, A). The amount of CPDs was significantly lower in PL-treated skin compared with PL-untreated skin at 24 hours ($P < .001$) (Fig 3, B). There was a significant reduction in epidermal proliferation seen by Ki67 immunoreactivity in PL-treated versus untreated skin at 72 hours ($P < .001$) (Fig 3, C). PL administration also resulted in a significant reduction of tryptase-positive mast cells in the papillary dermis compared with PL-untreated skin 24 and 72 hours ($P \leq .05$) (Fig 3, D). Although not statistically significant, PL treated skin showed preservation of Langerhans cells per square millimeter of epidermis in response to UVR when compared with PL-untreated skin at 72 hours. In addition, Langerhans cells in PL-untreated skin were increased in size and showed a loss of dendritic

morphology, whereas in PL-treated skin these cells preserved their size and dendritic appearance. Finally, there was a decrease in the surface area occupied by microvessels in PL-treated versus untreated skin at 24 and 72 hours, although the difference was not statistically significant. Table I gives an overview of all histologic parameter counts and statistical results.

DISCUSSION

In this study we demonstrate that oral administration of only two doses of PL, a naturally occurring agent endowed with antioxidant properties, was able to lead to a significant decrease in erythema, sunburn cells, DNA damage, UV-induced epidermal hyperproliferation, and mast cell infiltration in human skin. Furthermore, the histologic data show a trend toward Langerhans cell preservation and reduced vasodilatation. PL is an extract from a fern plant grown in Central America, which has been used for centuries by Native Americans for the treatment of malignant tumors.¹⁵ Elaborate studies have shown that PL has antioxidative properties,^{16,17} immunomodulatory properties,²⁶⁻²⁸ and antitumoral activity.^{15,29} Based on anecdotal observations, PL has been used safely for more than 30 years for the treatment of inflammatory skin diseases.³⁰⁻³³

In this study PL decreased skin sensitivity to UVR after intake of only two doses. This decrease was mild but significant, and can be compared with the

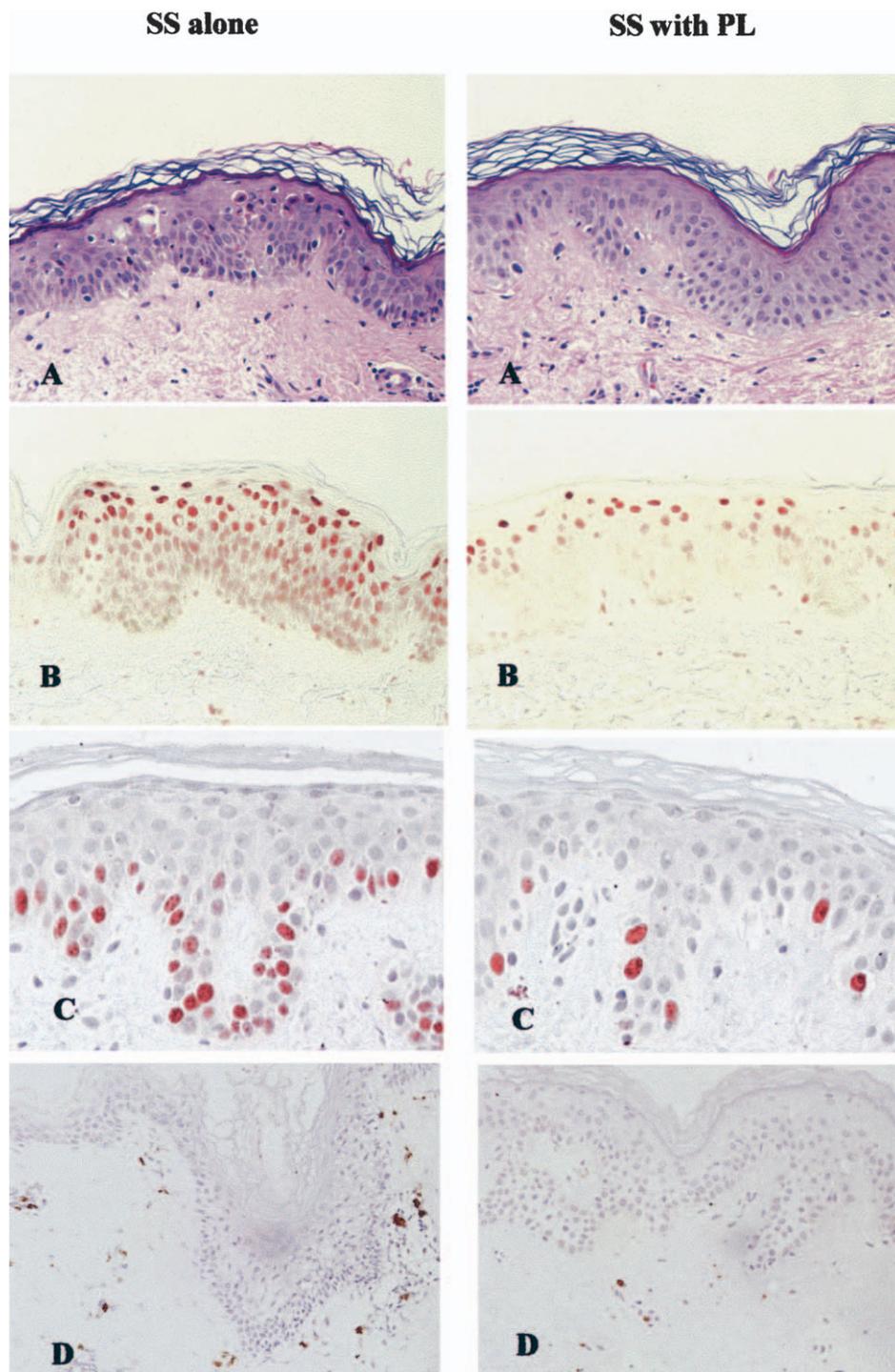


Fig 3. Histology from paired biopsy specimens of skin treated with UV radiation (SS) alone (left) and with *Polypodium leucotomos* (PL) (right). PL-treated skin shows: less sunburn cells, maturation disarray, microvesiculation, and vacuolization (A); less cyclobutane pyrimidine dimers (B); less epidermal proliferation (C); and less dermal mast cells infiltration (D).

effect obtained after repeated administration of other antioxidants.⁶⁻⁸ The clinically visible erythema reduction is histologically supported by the decrease in vasodilatation, which plays a role in erythema formation. PL does not have a specific absorption

peak or band in the UVB or UVA region,¹⁷ and we believe the photoprotective effect observed in this study to result from PL's antioxidative properties. The reduced epidermal hyperproliferation in PL-treated skin ($P < .001$), demonstrated by a lower

Table I. Quantitative overview of histologic parameters and *P* values between skin treated with UV radiation alone or with *Polypodium leucotomos*

Histologic parameter	UVR alone Mean (SEM)	UVR with PL Mean (SEM)
Sunburn cells/mm epidermis*	22.4 (2.03)	16.3 (2.9) [†]
CPD-positive cells/mm epidermis*	74.7 (4.58)	43.7 (4.03) [‡]
Mast cells/mm ² papillary dermis [§]	173.76 (19.36)	126.4 (14.72) [†]
Vasodilatation (% vessel surface area) [§]	2.38 (0.21)	1.94 (0.18)
% Ki-67-positive epidermal cells	38.85 (2.15)	25.94 (1.9) [‡]
Langerhans cells/mm ² epidermis	18.56 (5.76)	24.8 (6.08)

CPD, Cyclobutane pyrimidine dimer; PL, *Polypodium leucotomos*; UVR, UV radiation.

For each slide the number of CPD-positive cells, Langerhans cells, and mast cells were counted in at least 10 view fields (ocular grid, 0.0625 mm²; ×400). Data from biopsy specimens obtained at 24 hours,* at 24 and 72 hours,[§] and at 72 hours.^{||}

[†]*P* < .05; [‡]*P* < .001.

percentage of Ki67-positive epidermal cells (Fig 3, C), also shows PL's photoprotection, as an exposure to UVR is known to induce epidermal hyperproliferation.³⁴ It is believed that the resulting epidermal hyperproliferation is a protective response by skin against additional damage.³⁴ Fig 2 illustrates that the clinical photoprotective effect decreases as time between exposure and PL intake increases. It is known that skin antioxidants can be depleted by exposure to UVR,³⁵ but this depletion occurs locally at the irradiated skin site.³⁶ We performed each phototest separately while the rest of the skin was protected from UVR, therefore, our results suggest a rapid absorption and early bioavailability of oral PL, subsequently leading to an early photoprotection that lasts up to 2 hours after administration.

Although erythema is generally considered a marker for UVR damage, antioxidants decreasing erythema do not automatically provide photoprotection against long-term UVR effects such as skin (pre-)cancer formation.³⁷⁻³⁹ Currently, retinoids are the main oral chemopreventive agents successfully used in skin cancer prevention,^{40,41} but this occurs at doses that are associated with relevant side effects such as increases in cholesterol levels and mucocutaneous toxicity.^{42,43} Our histologic results show that the photoprotective effect of PL extends beyond just

decreasing the erythema reaction. CPDs are mutagenic and lead to carcinogenesis^{44,45} and subsequently their prevention is highly desirable. It is interesting that PL decreases CPDs as these lesions are not thought to result from oxidative damage.² However, DNA repair enzymes are susceptible to damage through oxidative stress,⁴⁶ and it is possible that the antioxidative properties of PL reduced this damage, allowing a better DNA repair and subsequently leading to lower numbers of CPDs. Others have found reduced amounts of CPDs in mice immediately after UVR exposure with topical antioxidant use, with a similar rate of CPD reduction afterward in treated and control groups and concluded that this could not be a result of enhanced repair.⁴⁷ However, DNA repair is known to be a cellular process that takes place continuously⁴⁸ and, therefore, we believe that enhanced repair might be one of the factors involved. Nonetheless, additional research work is warranted to further investigate this issue. Reduction of CPDs has been shown to reduce skin cancer development.⁴⁹ The significant decrease of CPDs (*P* < .001) by PL is, therefore, promising because this is the first report of an oral antioxidant to decrease DNA damage, and it suggests that PL might be able to prevent long-term skin damage such as skin cancer. In addition, PL significantly decreased sunburn cells (*P* < .05). Sunburn cells emerge when epidermal cells have suffered irreparable damage such as DNA damage,⁵⁰ and their numeric reduction shows the photoprotective ability of PL to decrease UVR-induced cell damage, most likely including DNA damage. We also found a trend toward Langerhans cell preservation in PL-treated skin. A study with a 10-week B-carotene supplementation lead to Langerhans cell preservation after physiologic doses of UVR.⁵¹ However, our study is the first to report of an oral antioxidant showing a tendency toward Langerhans cell preservation after exposure to two times the MED with intake of only two doses (*P* > .05). In PL-treated skin exposed to psoralen-UVA the Langerhans cell preservation was significant.¹⁹ Langerhans cell depletion is thought to play a role in the development of skin cancer, as it has been shown to be vital for the induction of tumor-specific immunity against UVR-induced tumors⁵² and the cells' presence has been shown to be indispensable for tumor rejection.⁵³ Finally, cell proliferation has been proposed as a predictive biomarker for carcinogenesis.⁵⁴ PL reduced epidermal proliferation as seen by the lower percentage of Ki67-positive keratinocytes. The combined histologic data is promising as it suggests that oral PL might help in the prevention of long-term UVR skin

damage, such as skin cancer. In vivo animal studies have shown that PL may be effective in decreasing the prevalence of UVB-induced skin cancer,²⁹ and carefully conducted studies in human beings will have to be performed to determine a long-term cancer-preventive effect in human beings. On the other hand, UVR also affects resident dermal cells. Among other dermal cells, mast cells are located in the papillary dermis, adjacent to blood and lymphatic vessels and in close proximity to peripheral nerves.⁵⁵ These cells may be implicated in UVB-induced skin immune suppression and photoaging.^{56,57} The ability of PL to decrease dermal mast cell infiltration might lead to a reduction of these two phenomena.

In conclusion, oral PL is an effective systemic chemophotoprotective agent as it leads to a significant decrease of UV-induced erythema, sunburn cell formation, DNA damage, epidermal hyperproliferation, and dermal mast cell infiltration. A trend toward Langerhans cell preservation and reduced vasodilatation was also seen. These short-term results are promising as they suggest that PL might be able to protect against long-term UV-induced skin damage. Further studies are needed to investigate this issue.

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