ORIGINAL ARTICLE

The impact of oral *Polypodium leucotomos* extract on ultraviolet B response: A human clinical study

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Background: There is a rationale for adding systemic photoprotective agents to the current photo-protection regimen.

Objective: This study was designed to objectively evaluate the molecular and photobiologic effects of oral administration of *Polypodium leucotomos* extract (PLE).

Methods: In all, 22 subjects with Fitzpatrick skin phototype I to III were enrolled. On day 1, subjects were irradiated with visible light, ultraviolet (UV) A1, and UVB (using 308-nm excimer laser). Evaluation was done immediately and 24 hours after irradiation. On days 3 and 4, irradiation and evaluation process was repeated after ingestion of PLE.

Results: Clinical assessments and colorimetry data showed a decrease in UVB-induced changes in 17 of 22 subjects post-PLE administration; histology findings demonstrated such a decrease in all 22 subjects.

Limitations: Only 2 doses of PLE were given. Furthermore, subjects with skin phototypes I to III only were studied.

Conclusion: The results suggest that PLE can potentially be used as an adjunctive agent to lessen the negative photobiologic effects of UVB. (J Am Acad Dermatol http://dx.doi.org/10.1016/j.jaad.2017.01.044.)

Key words: colorimetry; cyclobutane pyrimidine dimers; excimer laser; minimal erythema dose; *Polypodium leucotomos* extract; sunburn cells; ultraviolet.

Biologic effects of exposure to solar radiation include erythema, tanning, photoaging, and photocarcinogenesis. Most of these are a result of the direct and indirect effects of ultraviolet (UV) radiation. However, for those with skin phototypes IV to VI, visible light has been shown to induce intense and persistent pigmentation.[1-3]

Sunscreen and photoprotective clothing are effective photoprotective measures. However, neither of these are sufficiently used. Studies show that

sunscreens are applied at below half the tested concentration, and protective clothing is not worn because of the subject's concern about heat retention.[4,5] In addition, transparent organic and inorganic sunscreens do not prevent transmission of wavelengths in the visible spectrum.[6] Therefore, oral supplements with photoprotective properties may be helpful in reducing UV-induced injury when other photoprotective measures fail.

Polypodium leucotomos is a tropical fern grown in Central and South America; *Polypodium leucotomos* extract (PLE) has photoprotective benefits through its antioxidative, chemoprotective, immunomodulatory, and anti-inflammatory effects.[7] These properties are believed to be a result of several of the fern's polyphenols: caffeic, chlorogenic, ferulic, hydroxycinnamic, *p*-coumaric, and vanillic acids.[7] As an antioxidant, PLE enhances the ability of endogenous antioxidant systems to neutralize superoxide anions, lipid peroxides, and hydroxyl radicals, which are formed in the skin after exposure to UV and visible radiation.[8-10] In addition, lower levels of UV-induced cyclooxygenase-2 (COX-2) expression, p53 suppressor gene mutations, cyclobutane pyrimidine dimers, epidermal proliferation, sunburn cells, and inflammatory infiltrate are seen in vitro and in animal modules after PLE administration.[11,12] Cumulatively, these findings form the scientific basis behind previously reported human clinical trials evaluating the use of PLE in the treatment of vitiligo, melasma, and polymorphous light eruption, and in the prevention of skin cancer.[11,13-19]

Given the growing evidence supporting PLE's photoprotective effect and its broadening use in management of cutaneous disorders, this study was designed to quantify its effect on minimal erythema dose (MED) and clinical and histologic changes in those with skin phototypes I to III after exposure to visible light, UVA1, and UVB radiation, the latter using 308-nm excimer laser as the light source.

METHODS
Study subjects

In all, 22 healthy men and women with skin phototypes I to III were enrolled. The study was approved by the institutional review board of Henry Ford Hospital (no. 8386) in August 2013. Informed consent was obtained from all subjects. All guidelines from the Declaration of Helsinki were followed. Subjects with history of skin cancer, photoaggravated conditions, or medication were excluded. Those who were active tanners, pregnant, lactating, or known to have hypersensitivity to PLE were excluded. All subjects stated a willingness to limit participation in increased outdoor activities during the trial. A urine pregnancy test was performed on females reporting no menstruation in the prior 3 weeks. The first subject was enrolled on December 16, 2013.

Study design

On day 1, each subject was irradiated with very pure visible light, UVA1, and UVB on the left side of the back; the spectral output of the light sources is shown in Table I. On day 2, 24 hours after irradiation, assessments including clinical photography, Investigator Global Assessment (IGA) of erythema and pigmentation for each site, MED, colorimetry, and skin biopsies were performed. MED was defined as trace erythema within the irradiated area.[20] This process of irradiation and assessment was also repeated on days 3 and 4, respectively. However, on day 3 subjects ingested 240 mg of PLE, obtained from Ferndale Laboratories (Ferndale, MI), 2 hours and 1 hour before irradiation (ie, 480 mg total); irradiation was performed on the right side of the back.

Light sources and irradiation. The visible light source was Fiber-Lite model 180 (Dolan-Jenner Industries, Boxborough, MA) with a 150W EKE lamp with filter GG400/3mm (Schott North America Inc, Duryea, PA) and 3-mm hot mirror (Andover Corp, Salem, NH).

The UVA1 light source was Hamamatsu LightingCure UV Spot Light 200, 200W (Hamamatsu Photonics, Bridgewater, NJ). It emits from 240 to 400 nm; 2 filters were used, 3-mm WG-345 and 2-mm UG-11 (Schott North America Inc), which resulted in pure UVA1 (340-400 nm) radiation. The fluence rate for both light sources was adjusted to 25 and 225 mW/cm^2 for UVA1 and visible light, respectively, using an Oriel thermopile (Oriel, Stamford, CT). For irradiation, a liquid light guide with an 8-mm diameter was used for both visible light and UVA1 light source. Four visible light doses

CAPSULE SUMMARY

- A systemic agent with photoprotective properties would be a useful addition to current topical sun-protective modalities.

- Oral *Polypodium leucotomos* extract exhibited molecular and photobiologic protective effects against ultraviolet B.

- Oral *Polypodium leucotomos* extract may have potential as an adjunctive photoprotective agent.

> **Abbreviations used:**
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> COX-2: cyclooxygenase-2
> IGA: Investigator Global Assessment
> MED: minimal erythema dose
> PLE: *Polypodium leucotomos* extract
> UV: ultraviolet

(80, 160, 320, and 480 J/cm^2), and 5 UVA1 doses (22, 27, 33, 39, and 47 J/cm^2) were administered (Table I); these dose ranges were used based on the results of previous studies.[3,21] The UVB source was a 308-nm excimer laser (Xtrac, Photomedex, Montgomeryville, PA). A spot size of 3.2 cm^2 was irradiated. Standard UVB MED testing technique was used, which involves administration of 6 doses (100, 150, 200, 250, 300, and 350 mJ/cm^2) on the subject's back and assessments made at 24 hours postirradiation. The same set of doses were administered on either side (left and right) of the subject's back. The left side of the back was irradiated on day 1, and pre-PLE assessments were made on day 2. The right side of the back was irradiated on day 3, after PLE ingestion. Post-PLE assessments were made on day 4.

Assessments

Clinical photography and IGA. Clinical photographs of the back of the subjects were taken, and IGA scores for erythema and pigmentation were assigned to each site. The same investigator assigned the IGA score pre- and post-PLE administration. The IGA scale used for this study, shown in Supplemental Table I (available at http://www.jaad.org), was developed by the study investigators at Henry Ford Hospital. An IGA score of 1 (trace erythema) was defined as the MED.

Colorimetry. It is a noninvasive objective assessment technique used to quantitatively assess erythema and pigmentation. The colorimeter consists of a spectrophotometer (Konica Minolta CM-2600d, Konika Minolta, Osaka, Japan), a xenon arc lamp, and a computer. The site to be assessed was uniformly illuminated with visible light and information from the reflectance spectra was expressed as L* (lightness to darkness), a* (green to red), and b* (blue to yellow) color parameters. The a* value helps to objectively assess the intensity of erythema. It was normalized against the corresponding value of the subject's adjacent normal-appearing non-irradiated skin a_o* by calculating the ratio of $a*/a_o*$, referred to as the relative erythema intensity. Subjects thus acted as their own control. At clinically perceptible trace erythema, an IGA of 1, the relative erythema intensity was 1.6 ± 0.3. Colorimetry measurements were performed for all sites during each visit.

Histology. Skin biopsies were performed on day 2 and day 4. On day 2, two 4-mm punch biopsy specimens were obtained from unirradiated, normal-appearing skin (control biopsy specimen) and the site of MED/trace erythema (left side of back). On day 4, another 4-mm punch biopsy specimen was obtained from the site of MED/trace erythema (right side of back). All 3 skin biopsy specimens were stained for markers of inflammation (COX-2), apoptosis (sunburn cells), DNA damage (cyclobutane pyrimidine dimers), and cell proliferation (cyclin D1 and proliferating cell nuclear antigen).

Immunohistochemistry. Biopsy samples were embedded in paraffin wax and sectioned at 5 μm. After deparaffinization and rehydration, the manufacturer's protocol was followed for antigen retrieval (antigen unmasking solution, Vector Laboratories, Burlingame, CA). Expose mouse- and rabbit-specific horseradish peroxidase/3,3;-diaminobenzidine detection immunohistochemistry kit (Abcam, Cambridge, MA) was used for immune-histochemical staining of different antigens. Additional sections running in parallel but with omission of the primary antibodies served as the negative controls.

Antibodies for proliferating cell nuclear antigen, Ki67, cyclin D1, and COX-2 were purchased from Abcam. Cyclobutane pyrimidine dimers were stained with antithymine dimer antibody from Kamiya Biomedical Company (Seattle, WA).

The sunburn cells were counted on the hematoxylin and eosin–stained slides. The stained sections were examined with an Olympus BX51 microscope fitted with an Olympus DP71 digital camera (Olympus America Inc, Center Valley, PA). The number of positive cells at ×40 magnification were counted (cyclobutane pyrimidine dimers, Ki67, proliferating cell nuclear antigen, and cyclin D1) and an average from 6 randomly selected and distinct fields was taken as the final score. The staining intensity for COX-2 was scored on a basis of 5 staining intensity levels (0 = negative, 1 = weak, 2 = moderate, 3 = strong, and 4 = very strong). An average score of intensities from 6 different fields at ×40 was calculated for each biopsy specimen. All specimens were read in a blinded manner.

Statistical analysis

Statistical analysis was performed to compare the MED values, IGA scores, relative erythema intensity, dose response slope, and histology findings of day 2, referred to as pre-PLE, with those on day 4, referred

Table I. Spectral output of light sources and doses used

Device	Visible light radiation source Fiber-Lite*	UVA1 radiation source Lightningcure†	UVB radiation source Excimer laser‡
Spectral distribution, %			
UVB (290-320 nm)	0.000003%	0.00006%	308 nm
UVA2 (320-340 nm)	0.0000007%	0.00008%	
UVA1 (340-400 nm)	0.00004%	97.9%	-
Visible light (400-700 nm)	95.8%	0.74%	-
Near infrared	4.17% (700-1600 nm)	1.37% (700-800 nm)	-
Doses, J/cm²	80, 160, 320, 480	22, 27, 33, 39, 47	0.10, 0.15, 0.20, 0.25, 0.30, 0.35

UV, Ultraviolet.
*Fiber-Lite model 180, Dolan-Jenner Industries, Boxborough, MA.
†Hamamatsu LightingCure UV Spot Light 200, 200W, Hamamatsu Photonics, Bridgewater, NJ.
‡Xtrac, Photomedex, Montgomeryville, PA.

Table II. Values for ultraviolet-induced biomarkers pre- and post-*Polypodium leucotomos* extract

Biomarker	Units	Non-UV-treated skin*	UVB-irradiated skin pre-PLE*	UVB-irradiated skin post-PLE*	Reduction after PLE	P value pre- vs post-PLE
Proliferating cell nuclear antigen	No of positive cells/×40 field	80.4 ± 17.8	134.7 ± 29.8	89.9 ± 23.6	83%	<.0005
Sunburn cells	No of positive cells/×40 field	8.2 ± 3.2	20.1 ± 9.0	11.0 ± 4.6	76%	.00024
Cyclobutane pyrimidine dimers	No. of positive cells/×40 field	10.0 ± 5.3	97.0 ± 30.2	69.5 ± 23.3	32%	.001
Cyclin D1	No. of positive cells/×40 field	37.1 ± 20.8	71.7 ± 37	42.2 ± 25	85%	.0052
COX-2	Average staining intensity	2.0 ± 0.6	3.8 ± 0.4	2.4 ± 0.05	78%	<.0005
Ki67	No. of positive cells/×40 field	8.8 ± 3.0	14.5 ± 3.4	8.6 ± 2.5	100%	<.0005

Mean = average of 6 fields/section at ×40.
COX-2, Cyclooxygenase-2; *PLE, Polypodium leucotomos* extract; *UV*, ultraviolet.
*Mean ± SD for 22 patients.

to as post-PLE. Percent reduction in biomarkers was assessed by the following (Table II):

$$\% \, Reduction = \frac{(mean \; value \; UVB \; irradiated \; pre \; PLE) - (mean \; value \; UVB \; irradiated \; post \; PLE)}{(mean \; value \; UVB \; irradiated \; Pre \; PLE) - (mean \; value \; non-UV \; treated)} \times 100$$

Comparisons were made using 2-tailed paired-sample *t* test. In cases where distributional normality was significantly violated, the nonparametric Wilcoxon signed-rank test was used because of the paired nature of the data. Statistical significance was set at *P* less than .05 and all analyses were done using software (OriginPro, Version 9.1, OriginLab Corp, Northampton, MA).

RESULTS

All subjects exhibited a baseline erythematous response to UVB irradiation, but limited to no response was observed after exposure to UVA1 and visible light. Thus, the remainder of this section will focus on results related to UVB irradiation sites. A decrease in UVB-induced changes was seen post-PLE administration. This decrease was detected by noninvasive clinical assessments, colorimetry, and histology.

Clinical photography and IGA

Seven of 22 subjects exhibited an increase in MED after PLE administration, as assessed by IGA. Fig 1 is a set of representative clinical photographs demonstrating an increase in MED for a subject from 100 mJ/cm² pre-PLE to 150 mJ/cm² post-PLE administration. The increase in the post-PLE MED values did not reach statistical significance (*P* > .05).

Pre PLE MED Site 1

Post PLE MED Site 2

Fig 1. Ultraviolet B response. Pre-*Polypodium leucotomos* extract (*PLE*) minimal erythema dose (*MED*) site 1 (*left*) and post-PLE MED site 2 (*right*); set of representative clinical photographs demonstrating an increase in MED for a subject from 100 mJ/cm^2 pre-PLE to 150 mJ/cm^2 post-PLE administration.
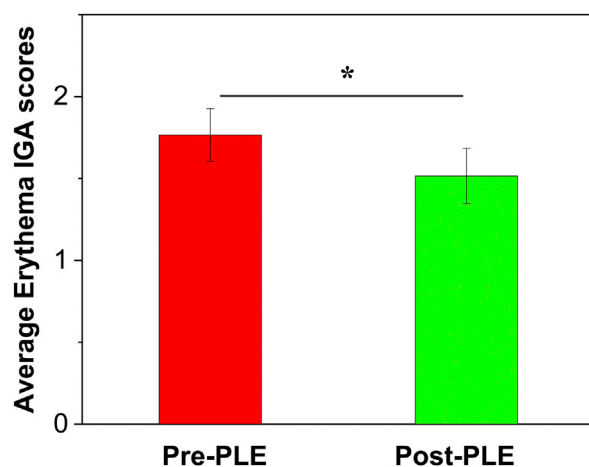
Fig 2. Ultraviolet B response. Average erythema investigator global assessment (*IGA*) scores. Mean ± SE of the mean. *Asterisk*, Statistically significant change ($P < .05$). *PLE, Polypodium leucotomos* extract.

Each subject's mean IGA score over the 6 combined doses was calculated from the left (pre-PLE) and the right (post-PLE) side. The post-PLE IGA scores were 19% lower than the pre-PLE ($P < .05$) (Fig 2).

Colorimetry

The projection of true color of L*, a*, and b* on the a* axis was analyzed for objectively assessing relative erythema intensity. This relative erythema intensity represented the number of folds that the erythema intensity of the irradiated site was higher compared to the adjacent normal nonirradiated skin. Each subject's mean relative erythema intensity over the 6 combined UVB doses was calculated from the left (pre-PLE) and the right (post-PLE) side. The post-PLE relative erythema intensity was 8% lower than that of the pre-PLE ($P < .05$) (Fig 3, *A*). This is a substantial difference considering the fact that the quantity compared was the number of folds change from baseline for pre- and post-PLE.

An increase in MED was detected for 7 of the 22 subjects by both colorimetry and IGA scores. In another 10 subjects, the MED (assessed by subjective IGA) remained the same post-PLE; however, there was a decrease in erythema intensity for a given dose of UVB as assessed objectively by colorimetry (Fig 3, *B*). Thus, 17 of 22 subjects demonstrated a decrease in UVB-induced changes after PLE administration. Four subjects remained the same with no change in MED or erythema intensity, and 1 subject had a decrease in MED.

Fig 3, *C*, is a representative of pre- and post-PLE relative erythema intensity for 1 subject as a function of UVB doses. It clearly reflects a decrease in the magnitude of the relative erythema intensity post-PLE and shows the difference in the slope with post-PLE being less steep. This slope for pre- and post-PLE as a function of UVB doses was analyzed for each subject and is referred to as the dose response slope. The dose response was found to be linear in the dose range of 0.5 to 2.8 MED. In this domain, the average post-PLE dose response slope was 18% lower than the pre-PLE ($P < .05$).

Histology

Studies were conducted to evaluate the effect of PLE on biomarkers associated with UV damage. These included parameters associated with DNA damage and apoptosis (sunburn cells and cyclobutane pyrimidine dimers), inflammation (COX-2), and proliferation (cyclin D1, Ki67, and proliferating cell nuclear antigen). There was a significant reduction in the deleterious effects of UV radiation on all of these biomarkers ($P < .05$) (Fig 4 and Table II). For cyclobutane pyrimidine dimers, the improvement was 32%. For the other biomarkers, the improvement ranged from 76% to 100%.
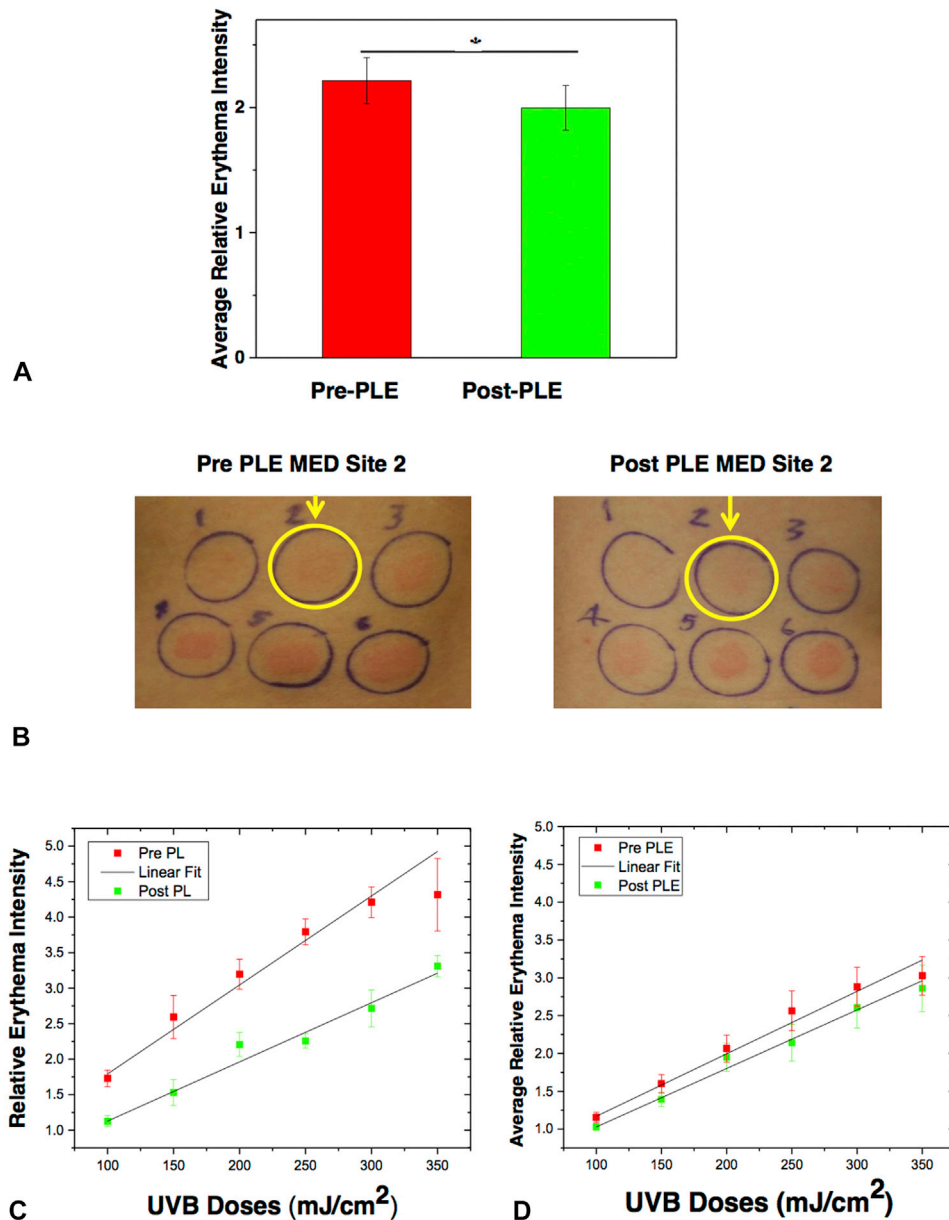
Fig 3. Ultraviolet (*UV*)B response. **A**, Average pre- and post-*Polypodium leucotomos* extract (*PLE*) relative erythema intensity. Mean ± SE of the mean. Asterisk, Statistically significant change ($P < .05$). **B**, Pre-PLE minimal erythema dose (*MED*) site 2, 150 mJ/cm^2 (*left*), post-PLE MED site 2, 150 mJ/cm^2 (*right*); set of representative clinical photographs demonstrating the decrease in erythema intensity although no change in MED. **C**, Pre- and post-PLE relative erythema intensity as a function of UVB dose for 1 subject. Mean ± SD. The pre-PLE MED for this subject was 100 (linear range 50−280 mJ/cm^2) and post-PLE MED was 150 (linear range 75-420 mJ/cm^2). Although all data points have been shown in the graph, slopes were calculated corresponding to the linear section only. **D**, Average pre- and post-PLE relative erythema intensity for all subjects as a function of UVB doses. Mean ± SE of the mean.

DISCUSSION

PLE has objective measurable suppressive effects on UVB-induced erythema within 2 hours of administration, which provides further insight on the photoprotective effects of PLE. PLE did exhibit significant chemoprotective and anti-inflammatory properties against UVB-induced damage as indicated by a decrease in the IGA scores, relative erythema intensity, and associated biomarkers after PLE administration.

Lighter-skinned individuals exhibit a steep dose response slope, implying that a small increase in dose will result in a considerable increase in

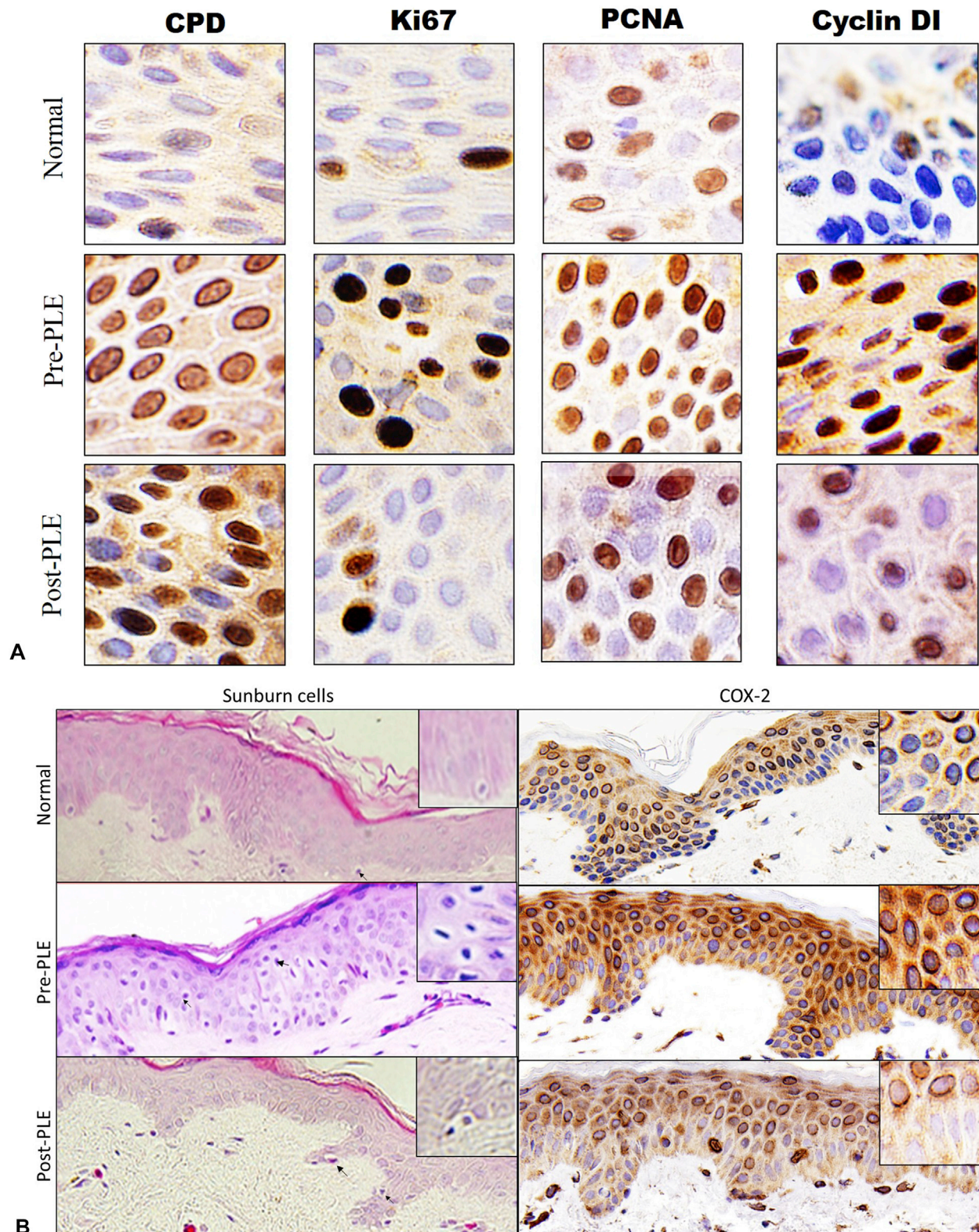**Fig 4.** Ultraviolet (UV)B response. Changes in biomarkers of UV radiation exposure before and after *Polypodium leucotomos* extract (*PLE*). **A**, Stained nuclei for cyclobutane pyrimidine dimers (*CPD*), Ki67, proliferating cell nuclear antigen (*PCNA*), and cyclin D1. **B**, Sunburn cells and cytoplasmic staining for cyclooxygenase-2 (*COX-2*). Representative photomicrographs. (Original magnifications: ×20.)

erythema. On the other hand, darker-skinned individuals have been shown to have a relatively flatter/less steep dose response slope indicating the need of larger dose increments to induce differences in erythema.[22] The less steep post-PLE dose response slope in our study (Fig 3, *C*) indicates that PLE had induced tolerance to UVB radiation, shifting the subject's response toward that of a higher/darker skin phototype. Thus, an individual's photobiologic responses (clinical and molecular) to UV radiation across a spectrum of dosages, which we term one's "photocapacity," improves after PLE ingestion.

Molecular damage has been reported to have a linear association after subjects were exposed to 0.5 to 3 MEDs of solar-simulating radiation[23]; our study found similar linear association for relative erythema intensity and UVB doses (Fig 3, *D*). This demonstrates a potential correlation between the colorimetry outcome of relative erythema intensity and molecular damage, suggesting the possibility of using colorimetry to serve as a noninvasive surrogate for quantification of cyclobutane pyrimidine dimers formation.

Our results, and those of previous studies, showed that PLE is a promising adjunctive method of oral photoprotection to traditional methods of photoprotection, which includes seeking shade; wearing photoprotective clothing, wide-brimmed hats, and sunglasses; and using sunscreen on exposed areas. Although traditional photoprotection limits photon exposure, oral antioxidants and photoprotectants (such as PLE, green tea extract, and silymarin) reduce free-radical induced cellular damage and thereby may improve the biologic photocapacity of individuals.[24]

There are limitations to our study. Only 2 doses of PLE were given 2 hours before exposure to UVB radiation. Furthermore, to facilitate evaluation of erythema, only subjects with skin phototypes I to III were studied. The UVA1 and visible light doses administered could have been on the lower side for the skin phototypes included in the study to induce erythema and pigmentation responses. Thus, our investigation was not able to determine whether PLE has an effect on response to UVA1 or visible light.

Future objective clinical investigations on subjects with skin phototypes IV to VI are needed to determine the role of PLE in mitigating the undesired effects of visible light and UVA irradiation. In addition, studies assessing the optimal dosage and timing of administration are needed to determine the maximum potential systemic photoprotective effects of PLE.

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Supplemental Table I. Investigator global assessment description of erythema and pigmentation

IGA	Erythema
0	Clear of erythema
1	Trace erythema
2	Visible, not confluent erythema, no sharp borders
3	Confluent erythema with 4 sharp borders
4	Intense erythema

IGA	Hyperpigmentation
0	Clear of hyperpigmentation
1	Almost clear of hyperpigmentation
2	Mild but noticeable hyperpigmentation
3	Moderate hyperpigmentation (medium brown in quality)
4	Severe hyperpigmentation (dark brown in quality)
5	Very severe hyperpigmentation (very dark brown, almost black in quality)

IGA, Investigator global assessment.