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Predominant effects of *Polypodium leucotomos* on membrane integrity, lipid peroxidation, and expression of elastin and matrixmetalloproteinase-1 in ultraviolet radiation exposed fibroblasts, and keratinocytes

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KEYWORDS
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Summary ***Background:*** *Polypodium leucotomos* has been reported to have antioxidant, anti-inflammatory and photoprotective properties. Exposure of skin to ultraviolet (UV) radiation can lead to deposition of excessive elastotic material, reduction in collagen, and increased expression of matrix metalloproteinases (MMPs). ***Objective:*** The goal of this research was to determine the effects of *P. leucotomos* in the absence or presence of UVA or UVB radiation on membrane damage, lipid peroxidation, and expression of elastin and MMP-1 in fibroblasts and keratinocytes, respectively. ***Methods:*** Fibroblasts and keratinocytes, respectively, were irradiated by a single exposure to UVA (0.6, 1.8 or 3.6 J) or UVB radiation (0.75, 2.5 or 7.5 mJ), and then incubated with, or without, *P. leucotomos* (0.01, 0.1 and 1%) and examined for membrane damage, lipid peroxidation, expression of elastin (protein levels) and MMP-1 (protein levels or MMP-1 promoter activity). ***Results:*** UV radiation did not significantly alter membrane integrity, lipid peroxidation or MMP-1 expression, but increased elastin expression. *P. leucotomos* significantly improved membrane integrity, inhibited lipid peroxidation, increased elastin expression, and inhibited MMP-1 expression in both fibroblasts, and keratinocytes. The effects of *P. leucotomos* predominated in the presence of UVA or UVB in both fibroblasts and keratinocytes, respectively, with the exception of inhibition of MMP-1 protein levels in fibroblasts only in combination with UV radiation. ***Conclusion:*** Lower concentration of *P. leucotomos* (lower than 0.1%), may be beneficial in preventing photoaging by improving membrane integrity and inhibiting MMP-1, without increasing elastin expression. Higher concentration (greater than 0.1%) of *P. leucotomos* may reverse the loss of normal elastic fibers associated with intrinsic aging.

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1. Introduction

Ultraviolet spectral distribution of solar radiation includes the long-wavelength UV-A light (320–400 nm) and the short-wavelength UV-B light (290–320 nm), both involved in the degenerative changes in the skin related to aging and cancer. UV radiation causes direct or indirect DNA damage, formation of reactive oxygen species (ROS), and associated inflammatory response and damage to the extracellular matrix integrity [1–3].

Chronic exposure of human skin to UV radiation results in photoaging. The detrimental effects of photoaging are wrinkle formation, diminished structural integrity, and impaired wound-healing [2]. In photoaged skin there is degeneration of collagen, induction of MMPs, and accumulation of elastin and fibrillin [2,4].

Polypodium leucotomos is a tropical fern plant belonging to a natural order polypodianceae. *P. leucotomos* has potent antioxidant and anti-inflammatory properties [5–10]. *P. leucotomos* extract can prevent sunburn reaction, inhibit the photosensitized generation/reactions of ROS, and has been shown to be effective in the reduction of UV induced tumors [1,5–7].

The purpose of this study was to determine the effects of *P. leucotomos* on the membrane integrity, lipid peroxidation, and the expression of elastin and MMP-1 in skin fibroblasts and keratinocytes in the absence or presence of low doses of UVA or UVB.

2. Methods

2.1. Cell culture

Dermal human fibroblasts (gift from Dr Lan Hu, Oncogene Science, NJ) were cultured in complete Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine. Human epidermal keratinocytes (Cascade Biologics) were cultured in Epilife media containing growth supplements (Cascade Biologics). Fibroblasts or keratinocytes, in replicates of four, were either non irradiated, or irradiated with a single dose of UVA radiation (0.6, 1.8 or 3.6 J), or UVB radiation (0.75, 2.5 or 7.5 mJ) prior to treatment with, or without, 0.01, 0.1 or 1% *P. leucotomos* for 24 h. The UV radiation was via a four tubes UVA lamp with irradiance of 1E-03 W/cm^2 for sum of wavelengths 320–400 nm or a four tubes UVB lamp with irradiance of 4E-05 W/cm^2 for sum of wavelengths 282–320 nm.

The media was examined for lactate dehydrogenase release to determine membrane damage, lipid peroxides to determine lipid peroxidation, and the expression of elastin and collagenase. To determine the transcriptional regulation of MMP-1 by *P. leucotomos*, keratinocytes were transfected with the MMP-1 promoter-chloramphenicol acetyl transferase (CAT) plasmid (gift from Dr William Parks, University of Washington, St. Louis, MI), prior to the experimental treatments, and examined for CAT expression. *P. leucotomos* was a gift to Dr Salvador Gonzalez from Industrial Farmacetica Cantabria (Madrid, Spain).

2.2. Membrane integrity

Membrane integrity was determined by measuring lactate dehydrogenase (LDH) release from cells via the LDH assay (Sigma). LDH reaction mixture (substrate, enzyme and a tetraxolium dye) was added to aliquots of media (1:2 ratio), incubated at room temperature for 30 min, and the LDH mediated conversion of the tetraxolium dye to a colored product was measured spectrophotometrically at 490 nm.

2.3. Lipid peroxidation

Lipid hydroperoxides were measured by the K-Assay (Kamiya Biomedical Company). The assay is based on the reaction of lipid hydroperoxides with a methylene blue derivative in the presence of hemoglobin to yield methylene blue that can be detected spectrophotometrically. Reagent 1 (ascorbic oxidase and lipoprotein lipase) was added to media aliquots (1:1 ratio) and incubated for 10 min at 30 °C. Equal volume aliquots of reagent 2 (methylene blue derivative and hemoglobin) were subsequently added, and the color change determined spectrophotometrically at 660 nm.

2.4. Elastin, and MMP-1 protein levels

The elastin and MMP-1 protein levels were determined by indirect ELISA (ELISAmate, Kirkeguard and Perry). Aliquots of media were added to a 96-well plate and incubated overnight at 4 °C. The wells were blocked with bovine serum albumin, and then incubated with elastin or MMP-1 antibody (Elastin Products Co., Sigma) for 1 h at room temperature. The plate was washed thoroughly with wash buffer, incubated with secondary antibody linked to peroxidase for 1 h at room temperature, washed with wash buffer thoroughly, and subsequently incubated with peroxidase sub-

strate until color development which was measured spectrophotometrically at 405 nm.

2.5. MMP-1 promoter activity

Keratinocytes were transfected with the MMP-1 CAT plasmid (10 ug per 33 mm dish) via ESCORT (Sigma) and followed by experimental treatments after 24 h. Cells were lysed by repeated freeze/thaw cycles, and examined for CAT expression by direct ELISA (Boehringer, Mannheim).

2.6. Data analysis

The effect of UV or *P. leucotomos* on cells was analyzed relative to cells that were non-irradiated and not exposed to *P. leucotomos* (control), represented as 100% in figures. The effects of *P. leucotomos* on modulating UV effects on cells were analyzed relative to UV effect alone, represented as 100% in figures. The data were statistically analyzed by ANOVA and Student's *t*-test at the 95% confidence interval for significance.

3. Results

Similar results were obtained with all three doses of UVA or UVB treatments, with or without *P. leucotomos* treatments and hence data corresponding mostly to 1.8 J of UVA, and 2.5 mJ of UVB is being represented in this paper.

3.1. *P. leucotomos* improved cellular membrane integrity

UVA, and UVB radiation did not alter membrane integrity in fibroblasts or keratinocytes. Cellular LDH release was 100 and 106%, respectively, in UVA (1.8 J) and UVB (2.5 mJ) treated fibroblasts relative to control fibroblasts (100%) (Fig. 1a). Relative to control keratinocytes (100%), the cellular LDH release was 126 and 87%, respectively, from UVA (1.8 J) and UVB (2.5 mJ) treated keratinocytes (Fig. 1b).

P. leucotomos significantly inhibited cellular LDH release from fibroblasts at concentrations of 0.1% (54%) and 1% (32%), relative to control fibroblasts (100%) ($P < 0.05$) (Fig. 1c). Similarly, *P. leucotomos* significantly inhibited cellular LDH release from UVA, and UVB treated fibroblasts at concentrations of 0.1% (UVA: 46% of UVA respective control; UVB: 62% of UVB respective control) and 1% (UVA: 33% of UVA respective control; UVB: 60% of UVB respective control) ($P < 0.05$) (Fig. 1c).

P. leucotomos significantly inhibited cellular LDH release from keratinocytes at concentrations of 0.1% (51% of control) and 1% (45% of control) (Fig. 1d). However, *P. leucotomos* was not significantly inhibitory to cellular LDH release in irradiated keratinocytes, relative to UV effects alone (Fig. 1d).

3.2. *P. leucotomos* inhibited lipid peroxidation

UVA, and UVB did not significantly alter lipid peroxidation in fibroblasts or keratinocytes. Relative to respective controls (100%), the lipid hydroperoxide levels were 69 and 96% in fibroblasts irradiated with UVA (1.8 J), and UVB (2.5 mJ), respectively, and 89 and 99% in UVA (1.8 J), and UVB (2.5 mJ) exposed keratinocytes (Fig. 2a, b).

P. leucotomos inhibited lipid peroxidation in non-irradiated and irradiated fibroblasts, and keratinocytes (Fig. 2c, d).

In non-irradiated fibroblasts, *P. leucotomos* reduced lipid peroxides at 0.01% (70% of control), 0.1% (52% of control) and 1% (32% of control) ($P < 0.05$) (Fig. 2c). Also, *P. leucotomos* significantly diminished lipid peroxides in UVA, and UVB exposed fibroblasts at 0.1% (UVA: 31% of UVA respective control; UVB: 46% of UVB respective control) ($P < 0.05$) (Fig. 2c).

P. leucotomos reduced lipid peroxides significantly at 0.01% (77% of control), 0.1% (45% of control) and 1% (32% of control) in non-irradiated keratinocytes ($P < 0.05$) (Fig. 2d). *P. leucotomos* also significantly reduced lipid peroxides in UVA, and UVB treated keratinocytes at concentrations of 0.1% (UVA: 43% of UVA respective control; UVB: 45% of UVB respective control) and 1% PL (UVA: 31% of UVA respective control; UVB: 32% of UVB respective control) ($P < 0.05$) (Fig. 2d).

3.3. *P. leucotomos* stimulates elastin expression

Relative to control fibroblasts (100%), UVA treatment of fibroblasts significantly increased elastin expression (protein) to 130% ($P < 0.05$) whereas UVB inhibited elastin to 63% ($P < 0.05$) (Fig. 3a). UVA increased elastin expression in keratinocytes but UVB caused a dramatic and significant increase (234% of control keratinocytes) ($P < 0.05$) (Fig. 3b).

P. leucotomos increased elastin secretion in non-irradiated and irradiated fibroblasts, and keratinocytes at the higher concentrations (Fig. 3c, d).

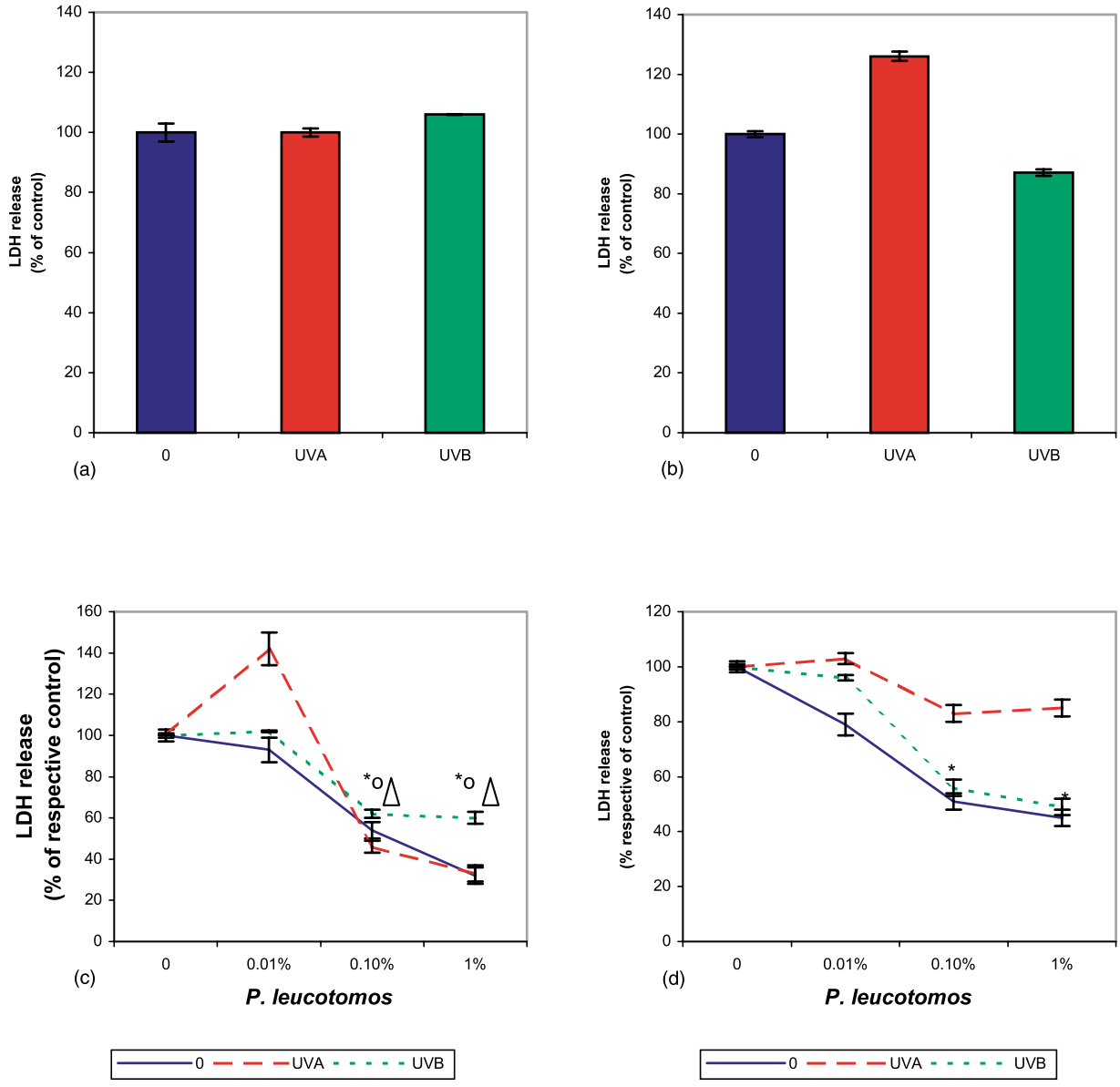

Fig. 1 Effects of UV radiation and/or *P. leucotomos* on cellular membrane integrity. UVA, and UVB effects on LDH release from fibroblasts (a), and keratinocytes (b). *P. leucotomos* and/or UV radiation effects on fibroblasts (c), keratinocytes (d); non-irradiated cells: solid blue line, (★) $P < 0.05$, UVA exposed cells: red dashed line, (○) $P < 0.05$, and UVB exposed cells: green dotted line, (△) $P < 0.05$. Error bars (a–d) represent standard deviation, $n = 4$.

P. leucotomos stimulated elastin significantly at 0.1% (155% of control) and 1% (350% of control) in fibroblasts ($P < 0.05$) (Fig. 3c). Similarly, *P. leucotomos* significantly increased elastin expression in UVA, and UVB exposed fibroblasts at 0.1% (UVA: 143% of UVA respective control; UVB: 136% of UVB respective control) and at 1% (UVA: 359% of UVA respective control; UVB: 264% of UVB respective control) ($P < 0.05$) (Fig. 3c).

P. leucotomos caused significant increase in elastin expression with 1% PL in the absence (137% of control), and presence of UVA (145% of respective UVA control) or UVB (166% of respective UVB control) ($P < 0.05$) (Fig. 3d).

3.4. *P. leucotomos* inhibits MMP-1 expression

UV exposure did not significantly alter MMP-1 expression in fibroblasts or keratinocytes. The MMP-1 expression (protein) in UVA, and UVB irradiated fibroblasts was 128 and 91%, respectively, of control fibroblasts (100%) (Fig. 4a). Relative to control keratinocytes (100%), the MMP-1 expression

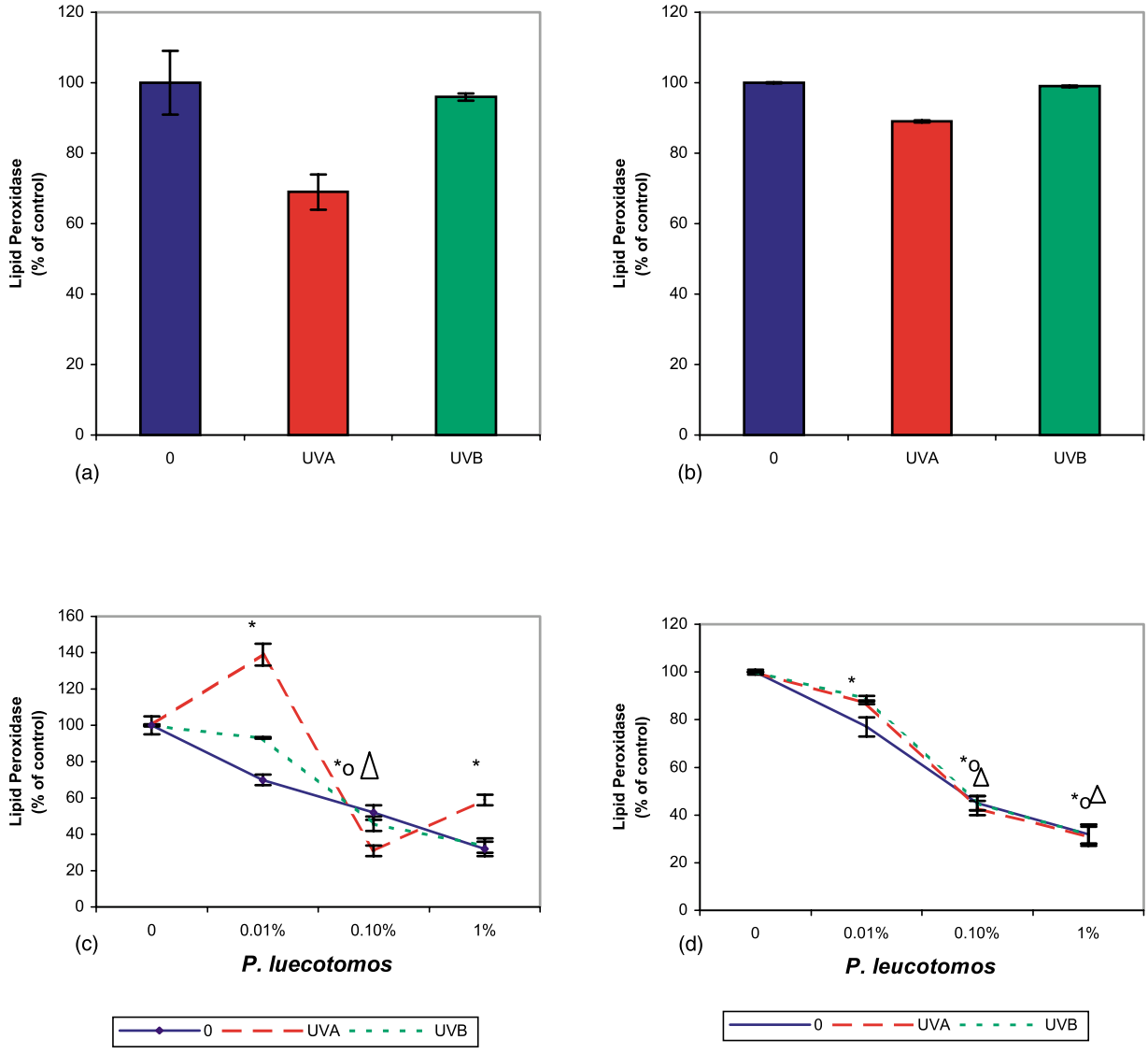

Fig. 2 Effects of UV radiation and/or *P. leucotomos* on lipid peroxidation UVA, and UVB effects on lipid peroxidation in fibroblasts (a), and keratinocytes (b). *P. leucotomos* and/or UV radiation effects on fibroblasts (c), keratinocytes (d), non-irradiated cells: solid blue line, (★) $P < 0.05$; UVA exposed cells: red dashed line, (○) $P < 0.05$; UVB exposed cells: green dotted line, (△) $P < 0.05$. Error bars (a–d) represent standard deviation, $n = 4$.

(promoter activity) was 103 and 87%, respectively, in UVA, and UVB treated keratinocytes (Fig. 4b).

P. leucotomos inhibited MMP-1 expression in fibroblasts, significantly at 1% in UVA exposed fibroblasts (55% of control), and significantly at all concentrations (0.01, 0.1, and 1%) in UVB exposed fibroblasts (42, 43 and 30% of control) ($P < 0.05$) (Fig. 4c).

P. leucotomos inhibited MMP-1 promoter activity in keratinocytes at 0.1% (79% of control) and 1% (74% of control) ($P < 0.05$) (Fig. 4d). It also significantly inhibited the MMP-1 promoter activity in keratinocytes in the presence of UVA, and UVB at 0.1% (UVA: 74% of UVA respective; UVB: 77% of UVB respective control) and at 1% (UVA: 71% of UVA respective control; UVB: 77% of UVB respective control) ($P < 0.05$) (Fig. 4d).

4. Discussion

Membrane damage and lipid peroxidation are induced by higher UVA doses of 100–200 J/cm^2 in fibroblasts [10,11]. Higher UVB doses of 0.75–3 J/cm^2 have been reported to increase membrane damage and lipid peroxidation in fibroblasts, but not in keratinocytes [12]. Under basal conditions, fibroblast and keratinocyte cultures release LDH and lipid peroxides. Keratinocytes have higher antioxidant content than fibroblasts, and secrete

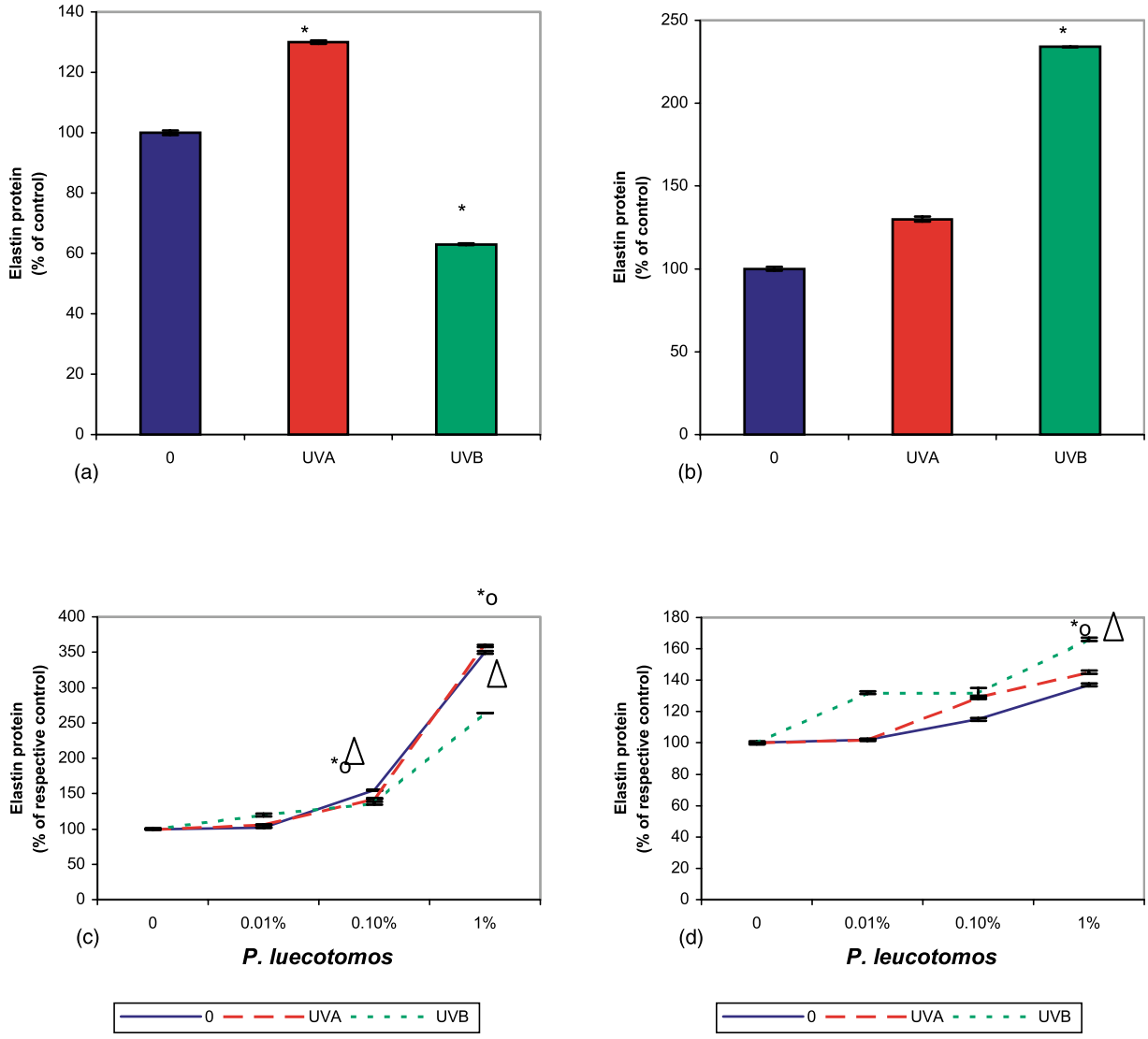

Fig. 3 Effects of UV radiation and/or *P. leucotomos* on elastin protein levels UVA, and UVB effects on elastin protein levels in fibroblasts (a), and keratinocytes (b), (★) $P < 0.05$. *P. leucotomos* and/or UV radiation effects on fibroblasts (c), keratinocytes (d), non-irradiated cells: solid blue line, (★) $P < 0.05$; UVA exposed cells: red dashed line, (○) $P < 0.05$; UVB exposed cells: green dotted line, (△) $P < 0.05$. Error bars (a–d) represent standard deviation, $n = 4$.

LDH and lipid hydroperoxides to a significantly lesser extent [13–15]. The lower doses of UVA, and UVB, used in our experiments, did not cause membrane damage or lipid peroxidation in fibroblasts or keratinocytes. *P. leucotomos* significantly inhibited membrane damage and lipid peroxidation in non-irradiated and irradiated fibroblasts and keratinocytes, indicating a protective effect.

UVA exposure of skin fibroblasts significantly stimulated elastin whereas UVB suppressed elastin expression in our experiments, as has been reported previously with higher doses of UVA or UVB [2,16,17]. UV exposure has been reported to increase elastin expression in epidermis, and keratinocytes have been suggested to be an additional source of elastin production in photoaged dermis [18]. Keratinocytes have been identified as the source of fibrillin in the microfibrils that form at the dermal–epidermal junction [19]. Also, cultured human keratinocytes express elastin in vitro [20]. However, the effects of UV exposure on elastin expression in cultured keratinocytes have not been previously reported. UVB, in particular, caused a dramatic and significant increase in elastin secretion in keratinocytes. The induction of elastin by UV in keratinocytes indicates that the keratinocytes may participate in the deposition of excessive elastin in the upper dermis in photoaging.

P. leucotomos significantly increased elastin expression in keratinocytes and fibroblasts at the higher concentrations. This increase may reflect

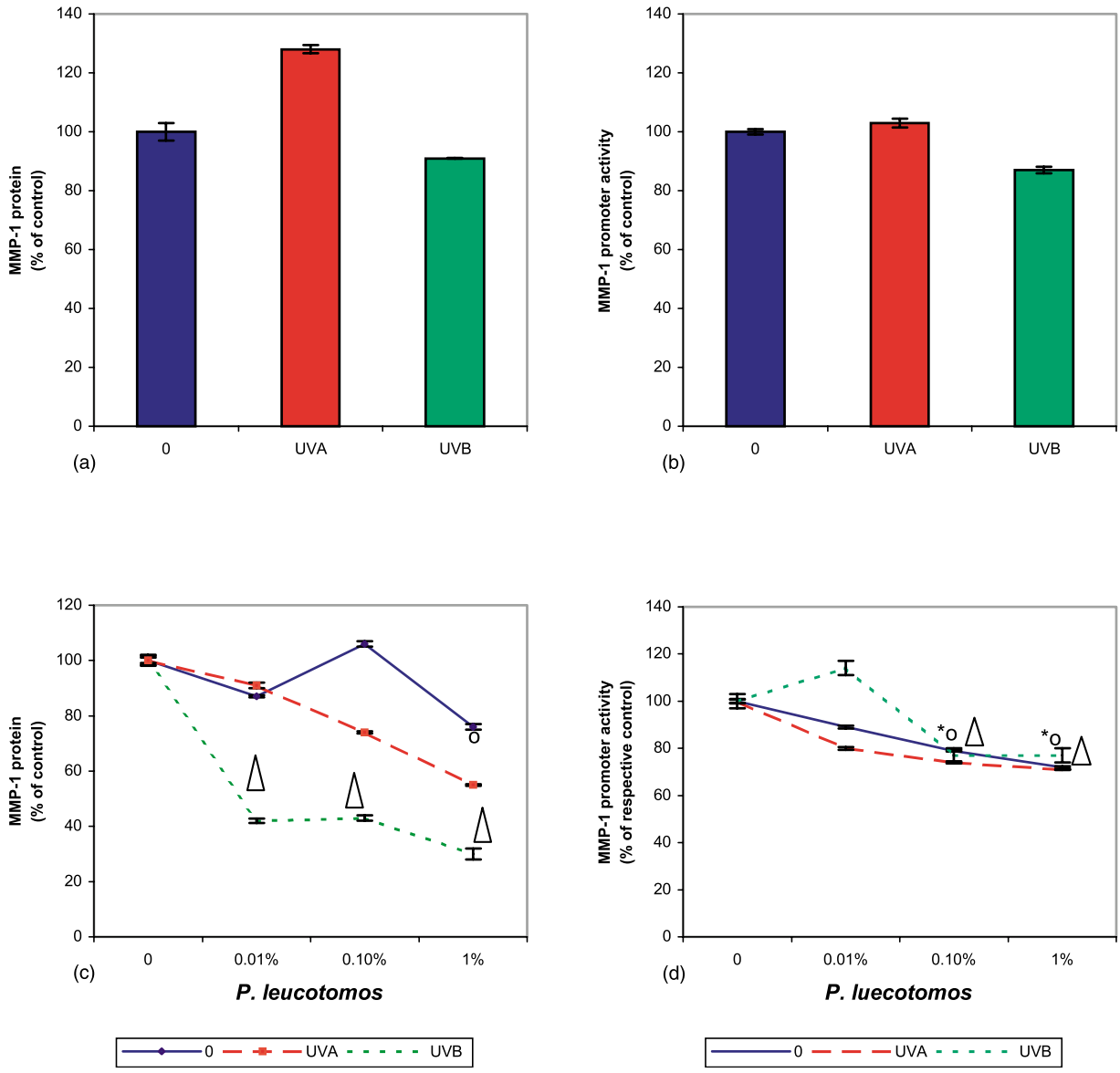

Fig. 4 Effects of UV radiation and/or *P. leucotomos* on MMP-1 Expression UVA, and UVB effects on MMP-1 protein levels in fibroblasts (a), and MMP-1 promoter activity in keratinocytes (b). *P. leucotomos* and/or UV radiation effects on fibroblasts (MMP-1 protein levels) (c), keratinocytes (MMP-1 promoter activity) (d), non-irradiated cells: solid blue line, (★) $P < 0.05$; UVA exposed cells: red dashed line, (○) $P < 0.05$; UVB exposed cells: green dotted line, (△) $P < 0.05$. Error bars (a–d) represent standard deviation, $n = 4$.

deposition of well-formed elastic fibers, and may be of relevance in stimulating elastin deposition in intrinsic aging. There is decline of elastin synthesis with intrinsic aging and a suggested pharmacological intervention is the identification of agents that can induce elastin biosynthesis [21]. The higher concentration of *P. leucotomos* has potential as a beneficial pharmacological agent to induce elastin biosynthesis in intrinsically aging skin.

UVA exposure at doses greater than 5 J/cm^2, in dose response experiments with 0–10 J/cm^2, has been reported to induce MMP-1 significantly, by transcriptional mechanisms, in fibroblasts and to inhibit MMP-1 mildly in keratinocytes [4,22–26]. UVB (0–100 mJ/cm^2) does not alter MMP-1 expression in keratincoytes or fibroblasts [4]. The lower UV doses used in our experiments did not alter MMP-1 expression in fibroblasts or keratinocytes. *P. leucotomos* inhibited MMP-1 expression (protein) in UV, particularly UVB, exposed fibroblasts indicating photoprotective effects. *P. leucotomos* inhibited MMP-1 promoter activity in nonirradiated and UV exposed keratinocytes.

Overall, the effects of *P. leucotomos* predominated with or without exposure to UVA or UVB radiation in both keratinocytes and fibroblasts, respectively. PL improved membrane integrity, inhibited lipid peroxidation, stimulated elastin expression and inhibited MMP-1 expression in both fibroblasts and keratinocytes. The only exception to the predominant effects of *P. leucotomos* was in the inhibition of MMP-1 expression only in combination with UV radiation, particularly UVB, in fibroblasts.

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