Arch Dermatol Res (2008) 300:69–80
DOI 10.1007/s00403-007-0818-x

ORIGINAL PAPER

Parthenolide-depleted Feverfew (*Tanacetum parthenium*) protects skin from UV irradiation and external aggression

Katharine Martin · Runa Sur · Frank Liebel · Neena Tierney · Peter Lyte · Michelle Garay · Thierry Oddos · Mike Anthonavage · Stan Shapiro · Michael Southall

Received: 8 June 2007 / Revised: 11 October 2007 / Accepted: 20 November 2007 / Published online: 11 December 2007

Abstract The skin is under continual assault from a variety of damaging environmental factors such as ultraviolet irradiation and atmospheric pollutants, and as organisms age the cumulative damage exceeds the capacity of endogenous antioxidant defenses resulting in chronic inflammation and premature aging. Botanical extracts such as Feverfew containing naturally occurring antioxidants could replenish the depleted cutaneous stores and perhaps forestall these degenerative changes. A parthenolide-depleted extract of Feverfew (PD-Feverfew), which was free of sensitization potential, was found to possess free radical scavenging activity against a wide range of reactive oxygen species and with greater activity than Vitamin C. In vitro, PD-Feverfew restored cigarette smoke-mediated depletion of cellular thiols, attenuated the formation of UV-induced hydrogen peroxide and reduced pro-inflammatory cytokine release. In vivo, topical PD-Feverfew reduced UV-induced epidermal hyperplasia, DNA damage and apoptosis. In a clinical study PD-Feverfew treatment significantly reduced erythema versus placebo 24 h post-UV exposure. Through the ability to scavenge free radicals, preserve endogenous antioxidant levels, reduce DNA damage and induce DNA repair enzymes, which can help repair damaged DNA, parthenolide-depleted extract of Feverfew may protect skin from the numerous external aggressions encountered daily by the skin and reduce the damage to oxidatively challenged skin.

Keywords Feverfew · Flavonoids · Oxidative stress · Parthenolide · Reactive oxygen species · Inflammation

Abbreviations

ROS	Reactive oxygen species
UVA	Ultraviolet A
UVB	Ultraviolet B

Introduction

The skin is the barrier which protects organisms from an environment that is becoming increasingly hostile. The daily onslaught of environmental factors such as solar UV radiation, coupled with recent increases in atmospheric pollutants and other external aggressors, provides an oxidative challenge that exacts a serious toll on skin health [6]. Endogenous antioxidants protect skin from the effects of oxidative stress; however, the levels of antioxidants in the skin decrease with age [22], thus resulting in less protection and a greater potential for skin damage [18]. With advancing age and thus total exposure, oxidative stress begins to overwhelm the skin's capacity to respond. This causes subtle changes that accumulate, accelerating the aging process and resulting in a state of chronic inflammation [40].

K. Martin · R. Sur · F. Liebel · N. Tierney · P. Lyte · M. Garay · M. Anthonavage · S. Shapiro · M. Southall
Johnson & Johnson Skin Research Center,
CPPW, a unit of Johnson & Johnson Consumer Companies, Inc.,
Skillman, NJ, USA

T. Oddos
Johnson & Johnson Skin Research Center,
CPPW, a unit of Johnson & Johnson Consumer Companies, Inc.,
Val de Reuil, France

M. Southall (✉)
Johnson & Johnson Consumer and Personal Products Worldwide,
199 Grandview Road, Skillman, NJ 08558, USA
e-mail: msoutha@cpcus.jnj.com

The primary environmental insult challenging the skin is solar ultraviolet (UV) irradiation, particularly its UVB component. UVB irradiation can induce many pathophysiological effects in the skin such as formation of reactive oxygen species (ROS) [34], DNA damage by thymine dimer formation [29, 48], lipid peroxidation [31, 32], dermal matrix protein degradation [40], alterations in membrane permeability [14], induction of pro-inflammatory mediator release [4, 42] and induction of apoptosis [3]. It has been known for decades that ROS are produced in the skin following UV irradiation [34] and are major mediators of oxidative damage to DNA and other cell constituents. These cellular changes induced by UV irradiation make solar exposure, particularly UVB, responsible for the induction of nonmelanoma skin cancer [27].

Other external aggressors that the skin is exposed to, such as cigarette smoke, mediate oxidative stress in human tissues by depleting intracellular thiols such as glutathione [30, 37]. This results in pathophysiological effects such as a decrease in phagocytic cell chemotaxis, increase in epithelial cell permeability and pro-inflammatory cytokine release, prevention of epithelial cell repair processes [25, 41, 50] and finally cell death. Smoke-induced oxidative stress can lead to premature skin aging, which is phenotypically similar to photoaged skin [38, 45].

Much attention has recently been focused on naturally occurring antioxidants, in particular botanical extracts, that provide effective protection from UV-induced damage [1]. In addition to the well-described effect of dietary beta-carotene, other carotenoids have shown effectiveness in counteracting the effects of UVB. Oral administration of a combination of the antioxidants, ascorbic acid and alpha tocopherol in humans significantly reduced both the sunburn reaction to UVB and the formation of thymine dimers [36].

Feverfew is an aromatic perennial, widely cultivated throughout Europe, which has been used for centuries as a source of herbal remedies. There are early references to its use for relieving fever, headache and migraine, as well as menstrual problems, stomachache, toothache and insect bites [13, 16]. Feverfew exhibits anti-inflammatory activity and parthenolide, a sesquiterpene lactone and an active constituent of Feverfew, has been shown to be responsible for many of its anti-inflammatory effects [20, 24]. However, parthenolide is a potent skin sensitizer and therefore would not be appropriate for topical applications [15, 35]. To eliminate the risk of skin sensitization from Feverfew, we developed a parthenolide-depleted extract of Feverfew (PD-Feverfew) and determined the effectiveness of PD-Feverfew in mitigating the effects of external aggression-induced oxidative stress in the skin.

Materials and methods

Depletion of parthenolide from feverfew

Extracts of *Tanacetum parthenium* with a reduced content of α-unsaturated γ-lactones, particularly parthenolide, were obtained by differential solvent extraction and subsequent elution on basic resins of Feverfew biomass [8]. Whole Feverfew contained $0.72\% \pm 0.08$ parthenolide as a percent of dry weight, and the parthenolide-depleted extract of Feverfew that was obtained contained $0.026\% \pm 0.001$, approximately 30 times less parthenolide as a percent of dry weight compared to whole Feverfew.

Cell culture

Primary normal human keratinocytes and primary normal human dermal fibroblasts were obtained from Cascade Biologics (Portland, OR, USA) and were cultured in Epilife growth media supplemented with human keratinocyte growth supplement.

Determination of free radical scavenging activity

The antioxidant scavenging capacity of Feverfew against the peroxyl radical (ORAC), hydroxyl radical (HORAC), peroxynitrite radical (NORAC), and the ferric reducing power of Feverfew (FRAP) were determined by Brunswick Laboratories (Norton, MA, USA) as previously described [19, 33]. $ORAC_{hydro}$ reflects water-soluble antioxidant capacity and the $ORAC_{lipo}$ is the lipid-soluble antioxidant capacity. Trolox, a water-soluble Vitamin E analog, is used as the calibration standard and the ORAC result is expressed as micromole Trolox equivalent (TE) per liter.

Determination of cell viability

Cell viability was assessed by the release of lactate dehydrogenase (LDH), a marker of cytotoxicity, using a cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN, USA).

Assessment of cigarette smoke-induced effects

Normal human dermal fibroblasts seeded in six-well format transwell inserts (Corning Costar, Cambridge, MA, USA) were incubated with a medium containing various concentrations of Feverfew for 24 h prior to exposure to either placebo (mock) or cigarette smoke (1 cigarette, BASIC Full Flavor 100's cigarettes, Philip Morris, Richmond, VA, USA) for 10 min. Prior to smoke exposure, the medium above the inserts containing the Feverfew extract was removed, while the one below remained during the smoke

exposure. After exposure, the cells were incubated for another 24 h with the previous medium. The cells were washed twice with Dulbecco's phosphate-buffered saline and lysed in 200 µl lysis buffer (caspase-3 colorimetric assay kit; R&D Systems Inc., Minneapolis, MN, USA). Intracellular thiols were then measured by mixing 100 µl of the lysate with 60 µM monobromobimane (Molecular Probes, Eugene, OR, USA) and incubating at 37°C for 30 min. In the presence of thiols, the monobromobimane becomes fluorescent. Fluorescence was measured using a CytoFluor® Fluorescence Plate Reader (PerSeptive Biosystems, Framingham, MA) set with excitation wavelength at 360 nm and emission wavelength at 460 nm.

Determination of hydrogen peroxide formation in normal human keratinocytes

UV-induced hydrogen peroxide formation was determined using a modification of the method of El Hindi [11] and Wang [49]. Primary human keratinocytes were plated at 8,000 cells/well in a 96-well plate. After 48 h, the cells were incubated for 30 min with 5 µM of the hydrogen peroxide-sensitive fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H2DCFDA; Invitrogen Carlsbad, CA, USA). After incubation, the plate was rinsed to remove excess probe and Feverfew was added at the indicated concentrations. The plate was immediately read on a fluorescent plate reader set at wavelengths 485 nm excitation/530 nm emission to detect basal peroxide formation. The plate was then exposed to UV (1000W-Oriel solar simulator equipped with a 1 mm Schott WG 320 filter; UV dose applied 4.2 kJ/m^2 as measured at 360 nm) or to cigarette smoke as described above and read 60 min post-UV exposure.

Assessment of UV-induced mediator release from reconstituted epidermis

Reconstituted human epidermis (EPI-200-HCF) was purchased from MatTek Corporation (Ashland, MA, USA). After reception, epidermal equivalents were incubated with a phenol-free and hydrocortisone-free maintenance medium (MatTek Corporation, Ashland, MA, USA) at 37°C for 24 h. PD-Feverfew, 1%, was prepared in 70% ethanol/30% propylene glycol vehicle and 5 µl was applied topically for 2 h prior to exposure to ultraviolet light (1000W-Oriel solar simulator equipped with a 1 mm Schott WG 320 filter) with a maximum irradiance of 7.52 mW/cm^2. The UV dose applied was 65 kJ/m^2, as measured at 360 nm, 95% UVA and 5% UVB. After 24 h, the medium below each equivalent was collected and analyzed for secreted IL-1α by ELISA using a commercially available immunoassay multiplex kit (Upstate Biotechnology, Charlottesville, VA, USA) on a Luminex L100 (Luminex Corporation, Austin, TX, USA).

Determination of photoaging in hairless mouse skin

SKh-1 hairless mice (Charles River Laboratories; Wilmington, MA, USA) were housed individually in an environmentally controlled room with a 12 h light and 12 h dark cycle and allowed food and water ad libitum. The Institutional Animal Care and Use Committee at Johnson & Johnson approved all procedures used in these experiments. The UVR source was a bank of 8 Philips TL40W/12 Ultraviolet-B bulbs, peak irradiance at 310 nm (Bulbtonics, NY, USA). Bulbs were positioned 19 cm above the mice for 3 weekly exposures, which continued for 10 weeks. Each UVB dose was gradually increased over the first 5 weeks by increments of 0.5 minimal erythema doses (MED) to a final dose of 4.5 MED (0.07 J/cm^2). Irradiance was measured with an IL-1400 Research Radiometer (International Light, Inc., Newburyport, MA, USA) using a UVB sensor. Two groups of mice ($N = 9$) were exposed to UVB irradiation with a third group ($N = 6$) serving as unirradiated controls. One of the irradiated groups was treated with 100 µl of 1% (10 mg/ml) PD-Feverfew solution made up in 70% ethanol/30% propylene glycol vehicle applied to the skin pre- and post-irradiation. After 10 weeks, the mice were sacrificed by CO_2 inhalation and the dorsal trunk skin was excised for histology. Hematoxylin and eosin (H&E) staining of mouse skin sections was performed by Paragon BioServices, Inc. (Baltimore, MD, USA). Thirty measurements per animal were made to derive a mean value for epidermal thickness.

Induction of UV-induced DNA and cellular damage in porcine skin

Gottingen microswine (Marshall Farms, NY, USA) were housed in appropriately sized cages in an environmentally controlled room with a 12 h light and 12 h dark cycle and supplied with Purina mini-swine chow and water ad libitum. The Institutional Animal Care and Use Committee at Johnson & Johnson approved all procedures used in these experiments. Animal care was based on the "Guide for the Care and Use of Laboratory Animals", NIH Publication No. 85-23. UV irradiation was given with a Hamamatsu Lightning Cure 200 UV Spot Light Source (Photonics K.K., Japan) with a 50 W xenon lamp filtered with a 2 mm hot mirror, 1 mm UG 11 (Schott Glass) and 1 mm WG320 (Schott Glass) to meet COLIPA solar simulator compliance. The fluence rate was set at 4 mW/cm^2 and measured with a calibrated Oriel Thermopile Model 71767. The MED was then determined from the least amount of time required to induce uniform redness after 24 h. A 1%

(10 mg/ml) PD-Feverfew solution made up in 70% ethanol/30% propylene glycol vehicle was applied to the skin (4 µl/cm^2) followed by UV light exposure at different multiples of MED intensity. After 24 h, biopsy punches were obtained from skin for histology. T-T dimer staining of swine skin sections was performed by Paragon BioServices, Inc. (Baltimore, MD, USA) using primary antibodies from Affitech (Oslo, Norway). Apoptosis staining (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling, TUNEL) was performed by Paragon Bioservices using Terminal Transferase, and Biotin-16-dUTP from Roche Diagnostics (Indianapolis, IN, USA).

Assessment of DNA repair

Normal human keratinocytes in culture were incubated with 10 and 30 µg/ml of PD-Feverfew extract. The cells were harvested after 1 h and nuclear extracts containing the DNA repair enzyme were prepared. Nuclei were prepared by incubating cells in the lysis buffer (Hepes 10 mM, pH 7.9; $MgCl_2$, 1.5 mM; KCl 10 mM; TritonX-100, 0.5%; dithiothreitol 0.5 mM; protease inhibitor PMSF). The nuclei were recovered by centrifugation and then lysed in the nucleus lysis buffer (Hepes 10 mM, pH 7.9; MgCl2 1.5 mM; KCl 0.4 M; glycerol 25%; EDTA 0.3 mM; dithiothreitol 0.5 mM; protease inhibitor PMSF). After centrifugation to remove residual particles, 10 µl (5 mg/ml of protein) of nuclear extracts were applied to hydrogel glass slides (Perkin Elmer) containing 1 mg/ml plasmid DNA-bearing lesions induced by different types of agents such as UV (thymine dimers and 6-4 photoproducts formed by exposing plasmid DNA to UVC irradiaiton with a dose of 0.3 J/cm^2 at 2.5 mW/s emitted by a VL 15-C lamp (Bio-block Scientific, Illkirch, France), endoperoxide DDE (mostly 8-oxo-guanosine lesions formed by treating 1 mg/ml plasmid and 20 µl endoperoxide at 37°C in the dark for 2 h), psoralen (psoralen adducts formed by irradiating 1 mg/ml plasmid and 120 mM psoralen amine with 1.48 J/cm^2 UVA light for 10 min) or cisplatin (cisplatin adducts formed by treating 1 mg/ml plasmid and 15 mg/ml cisplatin at 37°C in the dark for 2 h). The slides were then incubated for 2 h in the presence of Cy5-dCTP, during which the lesions were repaired. After the reaction, the level of DNA repair was analyzed by the level of fluorescent base (Cy5-dCTP) integrated in the DNA during the reaction.

Clinical study of UV-induced erythema

Topical emulsion formulations containing Feverfew were evaluated versus placebo formulations. The clinical study was a placebo-controlled, randomized, double blinded study of 12 subjects including both male and female of Fitzpatrick skin phototypes II and III, conducted at Harrison Research Laboratories, Inc. of Union, New Jersey. An independent review board (New England Institutional Review Board, Wellesley, MA, USA) approved the study protocol and all clinical investigation was conducted according to the Declaration of Helsinki principles. Briefly, subjects were required not to have any clinically visible erythema on the target areas on the back, or any past history of phototoxic, photallergic or any other abnormal responses to sunlight, or sensitivities to cosmetics, toiletries or topical drugs. Test formulations were applied for once daily for 2 days followed by exposure to UVB irradiation at varying MED on randomized pre-designated sites on the back. The UVB irradiation was from a custom-made light source with a peak output at 313 nm and a half-power bandwidth of 30 nm. The UVB intensity was $0.95 \pm 20\%$ mW/cm^2, as measured with the International Light Inc. Phototherapy System including IL1400A radiometer S/N5400, with UVB type I response sensor (detector SEL240 #5436) and diffuser (W#4489 and filter UVB-1 #8224). The UVB irradiation period was based on each subject's MED, as determined immediately prior to the study. Erythema was evaluated approximately 24 and 48 h following irradiation, using a Minolta CR300 Chromameter (Konica Minota Photo Imaging USA, Mahwah, NJ) [5]. Test formulations were re-applied post-UV exposure once daily for 2 days following chromameter and clinical grading.

Data analysis

Data are presented as mean ± standard deviation (SD) of three independent experiments. Student's *t* test was used to compare the effects of thiol loss in fibroblasts, peroxide formation in keratinocytes, IL-1 release for skin equivalents and erythema reduction in UV-induced erythema clinical model. The significance for all tests was set at $P < 0.05$.

Results

PD-Feverfew has a broad-spectrum antioxidant activity

The ability of PD-Feverfew to exhibit direct antioxidant activity was first tested. PD-Feverfew was found to possess radical scavenging activity against a wide range of free radicals, including oxygen, hydroxyl, peroxynitrate and ferric radicals (Table 1). In comparison to the reference antioxidant, ascorbic acid (vitamin C), PD-Feverfew had a fivefold greater scavenging activity for oxygen and hydroxyl radicals than ascorbic acid, and threefold greater scavenging activity for ferric radicals than ascorbic acid. PD-Feverfew had the greatest scavenging activity against ferric radicals,

Table 1 Antioxidant activity of PD-Feverfew

Dose (%)	Sample ID	$ORAC_{lipo}$ (μmol TE/l)	$ORAC_{hydro}$ (μmol TE/l)	[a]$ORAC_{total}$ (μmol TE/l)	HORAC (μmol caffeic acid equivalent/l)	NORAC (μmol TE/l)	FRAP (μmol TE/l)
0.5	PD-Feverfew	12,852 ± 813	11,632 ± 1405	24,484	1,994 ± 92	985 ± 127	3,9033 ± 2,125
0.1	PD-Feverfew	2,374 ± 276	2,547 ± 132	4,921	499 ± 35	195 ± 26	465 ± 25
0.5	Ascorbic acid	2,272 ± 234	3,471 ± 369	5,743	386 ± 46	4,491 ± 420	1,2771 ± 783

ORAC analysis provides a measure of the scavenging capacity of antioxidants against the peroxyl radical, which is one of the most common reactive oxygen species found in the body. [a]ORAC total is the sum of both the lipophilic and hydrophilic ORAC values. $ORAC_{hydro}$ reflects water-soluble antioxidant capacity and $ORAC_{lipo}$ is the lipid-soluble antioxidant capacity. HORAC analysis provides a measure of the scavenging capacity of antioxidants against hydroxyl radicals. NORAC analysis provides a measure of the scavenging capacity of antioxidants against peroxynitrite radicals. FRAP analysis provides a measure of the ferric reducing power of antioxidants. Trolox, a water-soluble vitamin E analog, is used as the calibration standard and the ORAC result is expressed as micromole Trolox equivalent (TE) per liter. Data is represented as mean ± SD of three independent experiments

followed by oxygen, hydroxyl and peroxynitrate radicals, respectively. PD-Feverfew was also effective in scavenging superoxide anion as measured by using a superoxide dismutase (SOD) assay kit (Dojindo Molecular Technologies, Gaithersburg, MD, USA). The IC50 of SOD scavenging activity for PD-Feverfew was 2.74 ± 0.39 μg/ml and that for ascorbic acid was 34.8 ± 2.13 μg/ml. Thus, the SOD activity of PD-Feverfew was 13-fold greater than that of ascorbic acid.

PD-Feverfew protects cells against the effects of cigarette smoke

Cigarette smoke (CS) is the major cause of pulmonary emphysema. Cellular oxidative stress is one of the several toxic effects of CS. CS mediates oxidative stress by depleting cells of essential thiols such as glutathione in vitro [28] and in vivo [7]. To assess the role of PD-Feverfew on CS-mediated effects, human dermal fibroblast cells were exposed to CS in the absence or presence of different concentrations of PD-Feverfew. A 10 min exposure to the equivalent of one cigarette reduced the dermal fibroblast thiol content by about 50% (Fig. 1a). Preincubation with PD-Feverfew gave a stepwise reversal of this inhibition, which was statistically significant ($P < 0.05$) at concentrations of 10 μg/ml and 25 μg/ml when compared with the smoke-exposed control. At these concentrations, cellular thiol content was maintained at levels similar to those in the no-smoke-exposed control. Likewise, cellular toxicity as measured by lactate dehydrogenase (LDH) release was also dose dependently reversed by PD-Feverfew treatment (Fig. 1b). PD-Feverfew was also found to protect keratinocytes from CS-induced ROS formation (Fig. 1c). CS was found to increase ROS formation, in keratinocytes, and preincubation, and PD-Feverfew gave a dose-dependent reduction in ROS formation in keratinocytes, which was statistically significant ($P < 0.05$) at concentrations as low as 6 μg/ml.

PD-Feverfew protects against UV-induced damage in vitro

Another major insult we are exposed to in our everyday life is UV irradiation. Thus, we thought it to be important to ascertain whether PD-Feverfew plays any protective role against UV-induced cellular damage. It has been well established that ROS such as H_2O_2 and peroxyl radical are produced in the skin following UV irradiation [44] and are major mediators of oxidative damage to DNA and other cellular constituents. To assess the role of PD-Feverfew on UV-induced cellular damage, we determined whether PD-Feverfew had any effect on UV-induced hydrogen peroxide (H_2O_2) formation in normal human keratinocytes. A dose of 4.2 kJ/m^2 (at 360 nm) from a solar simulator increased intracellular hydrogen peroxide by 73% (from 173 to 298 units; Fig. 2a). Preincubation with PD-Feverfew at concentrations from 3.1 to 100 μg/ml attenuated this stimulation in a dose-related, statistically significant manner ($P < 0.05$). At concentrations greater than 10 μg/ml, the suppression was to levels below those in non-irradiated controls indicating that PD-Feverfew reduced the basal level of hydrogen peroxide present in keratinocytes.

UV irradiation has been shown to induce the release of various inflammatory cytokines such as IL-1α, IL-6 and TNF-α, which are involved in the pathophysiology of UV-induced inflammation [4]. We next looked at the effect of PD-Feverfew on UV-induced IL-1α release from reconstituted human epidermis. As shown in Fig. 2b, there was a low level of 129 ± 38 ng/ml IL-1α release from non-irradiated skin equivalents. After UV irradiation with 65 kJ/m^2, the amount of IL-1α increases by about sixfold to 737 ± 50 ng/ml. Pretreatment with PD-Feverfew did not change the basal level of IL-1α release, however significantly reduced UV-induced IL-1α release, which decreased from 737 ± 50 ng/ml to 294 ± 30 ng/ml, a 60% reduction over placebo-treated control skin equivalents. Additionally, post-treatment with PD-Feverfew reduced UV-induced IL-1α release by 40% over placebo-treated control skin

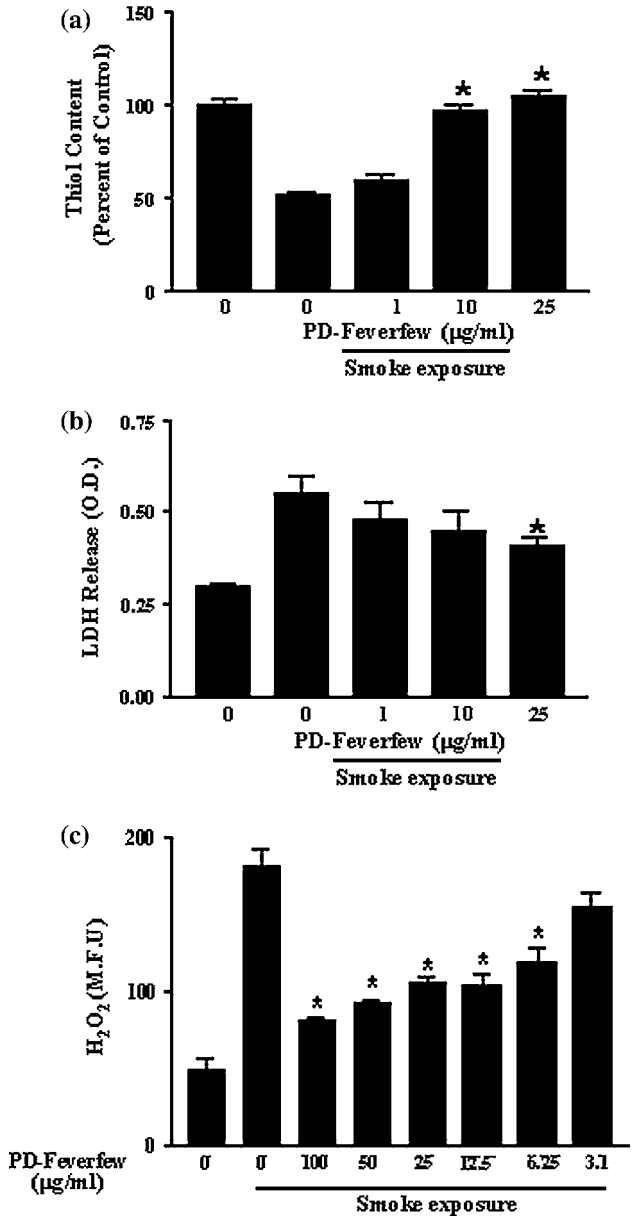

Fig. 1 PD-Feverfew reduces cigarette smoke-induced damage to normal human dermal fibroblasts and human keratinocytes. **a** Intracellular thiol content was measured and is represented as a percent of control. $^*P < 0.05$ compared with smoke exposed untreated control fibroblasts. **b** Lactate dehydrogenase release was measured and is represented as optical density (OD) units. $^*P < 0.05$ compared with smoke-exposed untreated control fibroblasts. **c** PD-Feverfew inhibits smoke-induced H_2O_2 production in primary human keratinocytes. Keratinocytes were treated with vehicle (placebo) or 1% PD-Feverfew and exposed to cigarette smoke. H_2O_2 production is represented as mean fluorescent units (MFU). $^*P < 0.05$ compared with smoke-exposed vehicle-treated control keratinocytes

equivalents. Pretreatment with PD-Feverfew significantly ($P < 0.05$) prevented UV-induced cellular cytotoxicity by reducing LDH release from 4.39 ± 0.36 to 2.78 ± 0.22 (Fig. 2d).

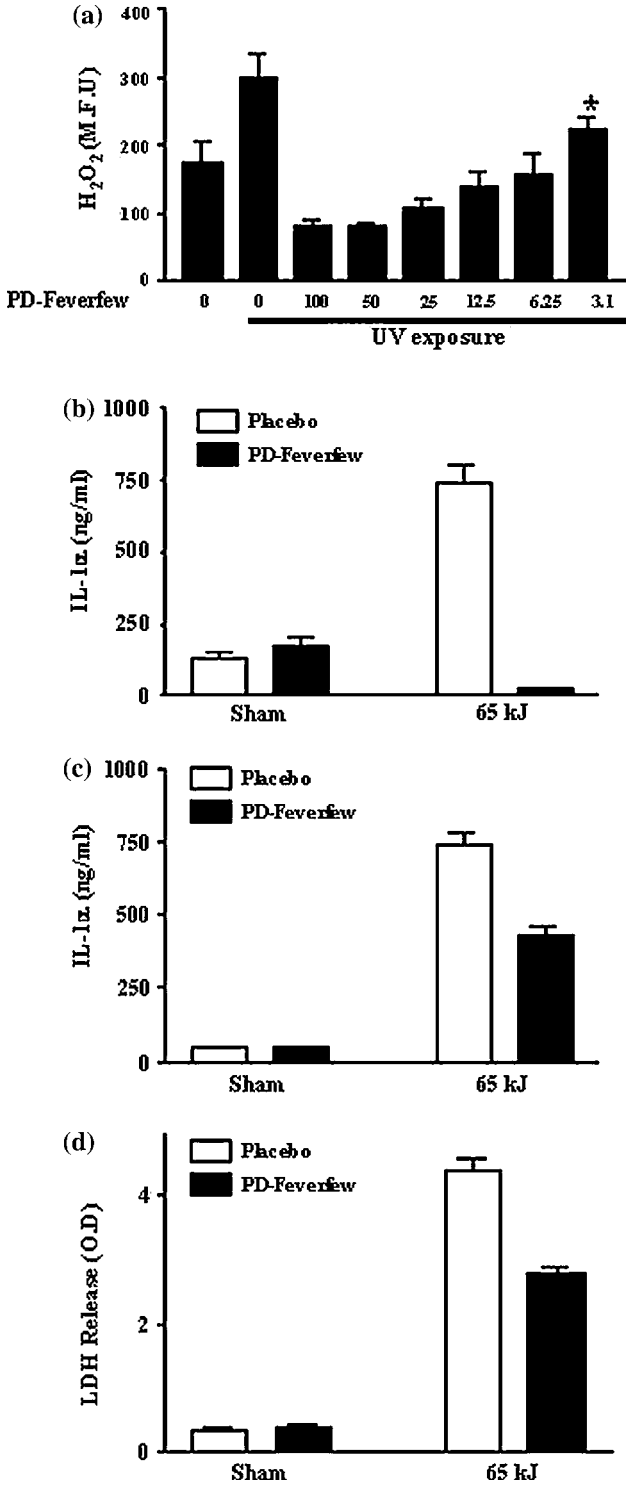

Fig. 2 PD-Feverfew reduces UVB-induced damage in vitro **a** PD-Feverfew inhibits UVB-induced H_2O_2 production in primary human keratinocytes. H_2O_2 production is represented as mean fluorescent units (MFU). $^*P < 0.05$ compared with UV-exposed vehicle-treated control keratinocytes. Reconstituted human epidermis (EPI-200-HCF) was pretreated with vehicle (placebo) or 1% PD-Feverfew for 2 h and exposed to UV irradiation **b** or post-treated with vehicle (placebo) or 1% PD-Feverfew immediately after UV irradiation **c** After 24 h, IL-1α (ng/ml) release was measured by ELISA. **d** Cell viability was assessed using release of lactate dehydrogenase (LDH) as a marker of cytotoxicity

PD-Feverfew protects against UV-induced damage in vivo

To determine whether PD-Feverfew exhibited any protective effects on UV-induced damage in vivo, we first looked at the effects of PD-Feverfew on photoaged mouse skin. Exposure of mouse epidermis to UV irradiation results in its pronounced thickening [21, 43] as seen in Fig. 3a. Application of 1% PD-Feverfew markedly attenuated the hyperplasia response. The values of epidermal thickness increased from 31.3 ± 7.0 to $62.3 \pm 9.7\ \mu$ following UVB and significantly ($P < 0.05$) decreased to $45.4 \pm 5.2\ \mu$ in the group pretreated with 1% PD-Feverfew (Fig. 3b).

One of the major adverse effects of UV irradiation is damage to DNA, so next we looked at the effects of PD-Feverfew on cellular DNA damage. Thymine dimer immunostaining of swine skin tissue left unexposed or exposed to UV irradiation with or without pretreatment with PD-Feverfew is presented in Fig. 4a. To eliminate the potential of PD-Feverfew to absorb UV irradiation, the surface of the skin was gently washed with water prior to UV exposure. The quantitative values for stain uptake are represented in Fig. 4b. One MED (160 mJ/cm^2) produced a tenfold increase in T-T formation. After pretreatment with 1% PD-Feverfew, T-T formation increased by only threefold, which was 48 and 35% lesser than the values in the untreated and vehicle-treated group, respectively (Fig. 4b). Likewise, after 2.5 MED, the T-T formation was stimulated about 14-fold and pretreatment with 1% PD-Feverfew afforded a significant protection by limiting the stimulation to 8.5-fold (data not shown).

Until cells reverse these types of lesions, either by light-dependent photorepair or nucleoside-excision repair. DNA transcription is blocked, which acts as a trigger for, among other things, programmed cell death or apoptosis [10, 17]. To investigate whether PD-Feverfew had any effects on UV-induced apoptosis, Gottingen microswine were left untreated or exposed to 1 MED UV irradiation in the absence or presence of 1% PD-Feverfew. TUNEL immunostaining of skin biopsies showed that UV exposure dramatically increased the number of TUNEL-positive apoptotic cells and pre- and post-treatment with PD-Feverfew reversed this effect with the epidermis, comparable to the untreated controls (Fig. 4c).

Since the reduced apoptosis could reflect either the activity of PD-Feverfew to inhibit UV-induced DNA damage from occurring or could occur by increasing the repair of UV- DNA damage, we examined the effect of PD-Feverfew on DNA repair processes. PD-Feverfew induced a dose-dependent increase in the enzymatic activity of DNA repair enzymes in human epidermal keratinocytes (Table 2). In addition to repairing CPD damage from UV, PD-Feverfew also reduced DNA damage caused by the chemotherapeutic agent, Cisplatin, and the photodynamic agent, Psoralen. PD-Feverfew was also found to induce the nucleotide excision repair process in untreated human epidermal keratino-

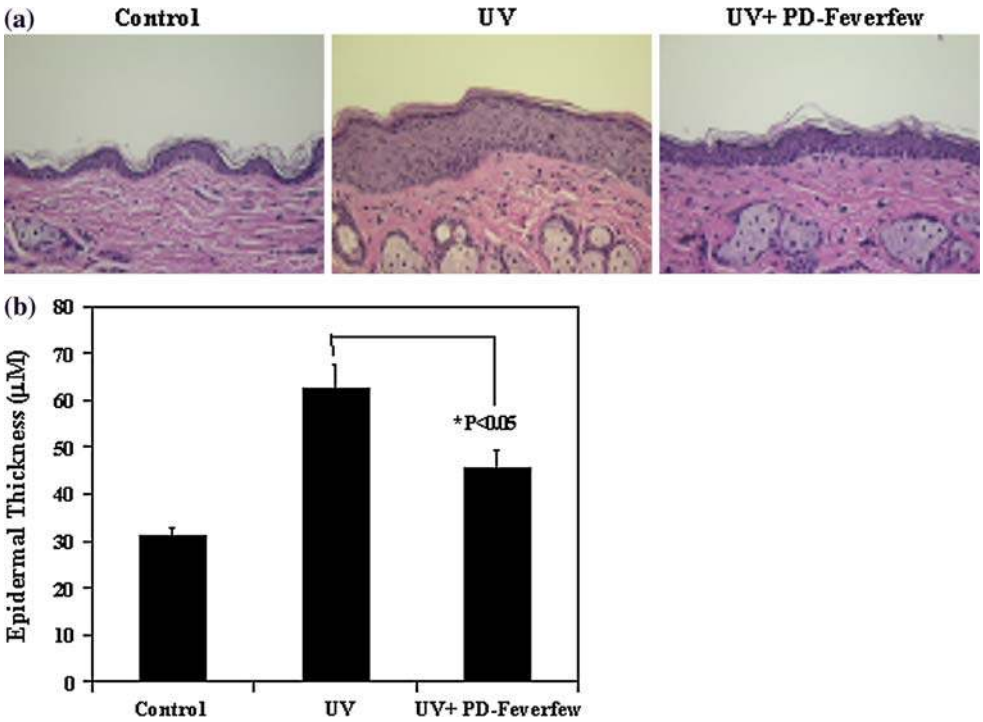

Fig. 3 PD-Feverfew reduces UVB-induced hyperplasia in mice. **a** SKh-1 mice were exposed daily to UVB light for 10 weeks and 1% PD-Feverfew was applied topically prior to and following UV exposure. Skin samples were stained with hematoxylin and eosin (H&E) and histologically evaluated with image analysis. **b** Representation of mean ± SD of epidermal thickness measured for the different groups

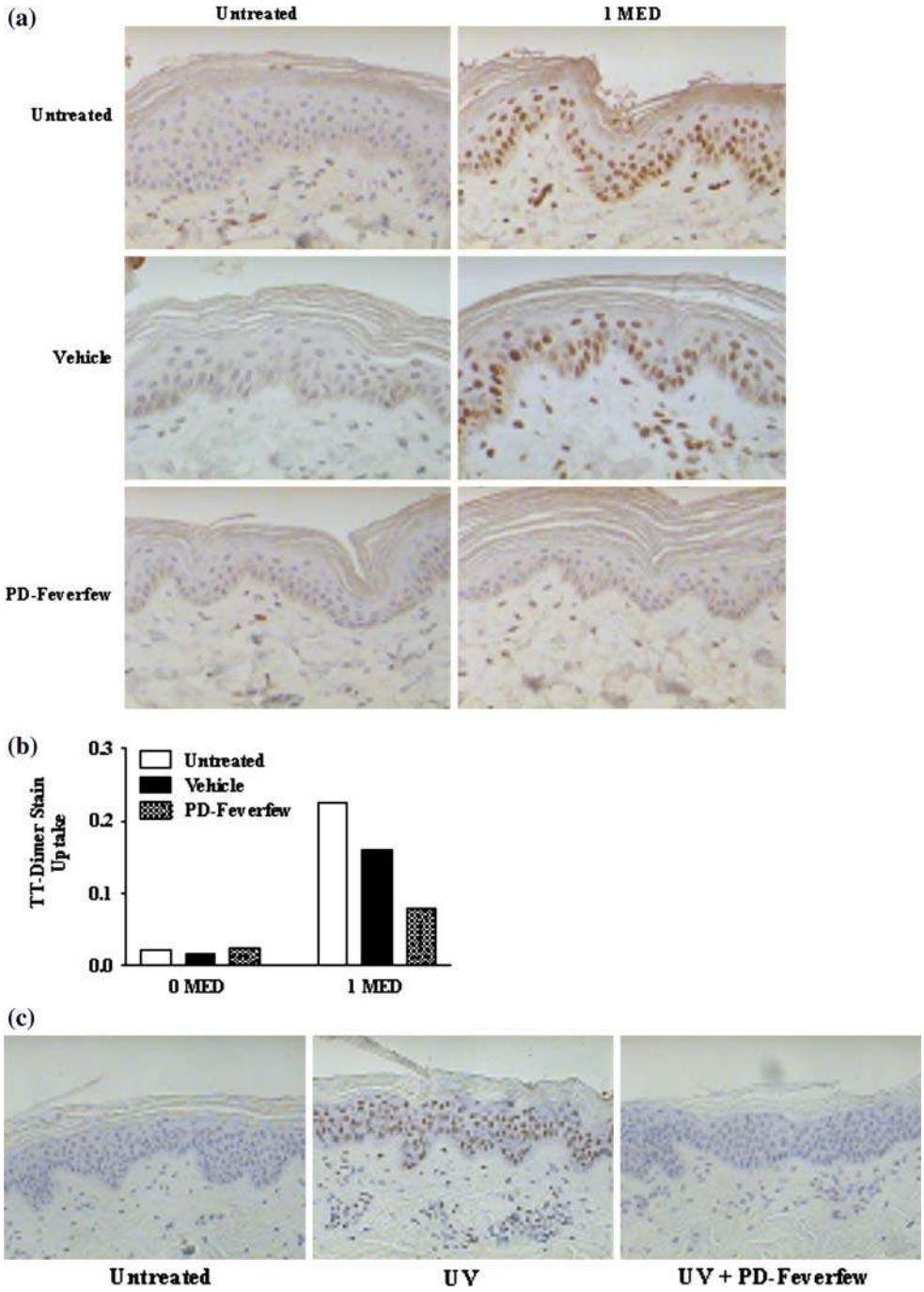

Fig. 4 PD-Feverfew reduces UV-induced DNA and cellular damage in porcine skin. Gottingen microswine were pretreated with vehicle or PD-Feverfew (1%) for 30 min prior to UV irradiation exposure of 1 MED (160 mJ/cm^2). After 24 h, biopsy punches of the skin were obtained and **a** T-T dimer staining was performed. **b** *Bar graph* representing T-T dimer stain uptake in the skin biopsy punches. **c** TUNEL immunostaining of skin biopsies was performed to determine apoptotic cells. Data represent one of three independent experiments

cytes, which could repair DNA damaged by intrinsic metabolic ROS formation.

PD-Feverfew reduces UV-induced erythema: a clinical study

To elucidate the effect of PD-Feverfew on UV irradiation-induced erythema, human subjects were exposed to 0.5, 1 and 1.5 MED UV irradiation. Figure 5a shows a representative cross-polarized photographic image of a subject at 24 and 48 h post-UVB irradiation, depicting UV-induced erythema that was further evaluated via clinical expert grading and instrumental assessment. As evident from Fig. 5a, at 24 and 48 h post-UVB irradiation, there is a marked reduction in visible erythema for the site treated with 1% PD-Feverfew formulation versus the placebo formulation. In the expert grader assessment, at 24 and 48 h post-UV irradiation, PD-Feverfew-mediated improvements in erythema relative to placebo were statistically significant ($P < 0.05$) at 1.0 and 1.5 MED UVB dose (Fig. 5b). Chromameter

Table 2 Effect of PD-Feverfew on DNA repair process

Lesion carried by DNA (treatment/lesion)	Control	PD-Feverfew 10 μg/ml	PD-Feverfew 30 μg/ml
Control (no lesion)	1637 ± 277	2172 ± 423	3579 ± 691
UVC (TT dimers)	2664 ± 693	3990 ± 579	5971 ± 1043
Endoperoxide (8-oxo-G)	1885 ± 506	2055 ± 341	5005 ± 2021
DDE (alkylated base)	6807 ± 2504	6584 ± 2038	17453 ± 5906
CIS (cisplatin adducts)	1203 ± 291	1968 ± 569	3011 ± 882
PSO (psoralen adducts)	2725 ± 357	3758 ± 1818	8251 ± 1984

Normal human keratinocytes were incubated with 10 and 30 μg/ml of PD-Feverfew. Nuclear extracts were obtained and applied to hydrogel glass slides (Perkin Elmer) containing plasmid DNA-bearing lesions induced by different types of agents such as UV (thymine dimers and 6-4 photoproducts), endoperoxide, DDE (oxidative lesions mostly 8-oxo-guanosine), cisplatin (cisplatin adducts) or psoralen (psoralen adducts). The slides were then incubated with Cy5-dCTP and the efficiency of DNA repair was analyzed by the level of fluorescent base (Cy5-dCTP) integrated in the DNA during the reaction, represented as mean fluorescent intensity ± SD representing three independent experiments

readings confirmed the improvements in erythema versus placebo at 24 and 48 h post 0.5–1.5 MED UV irradiation (Fig. 5c). Photo-absorbance studies conducted using a Labsphere confirmed that PD-Feverfew did not substantially absorb UVB (data not shown) and thus indicates that the reduction in erythema does not result from a non-specific interference.

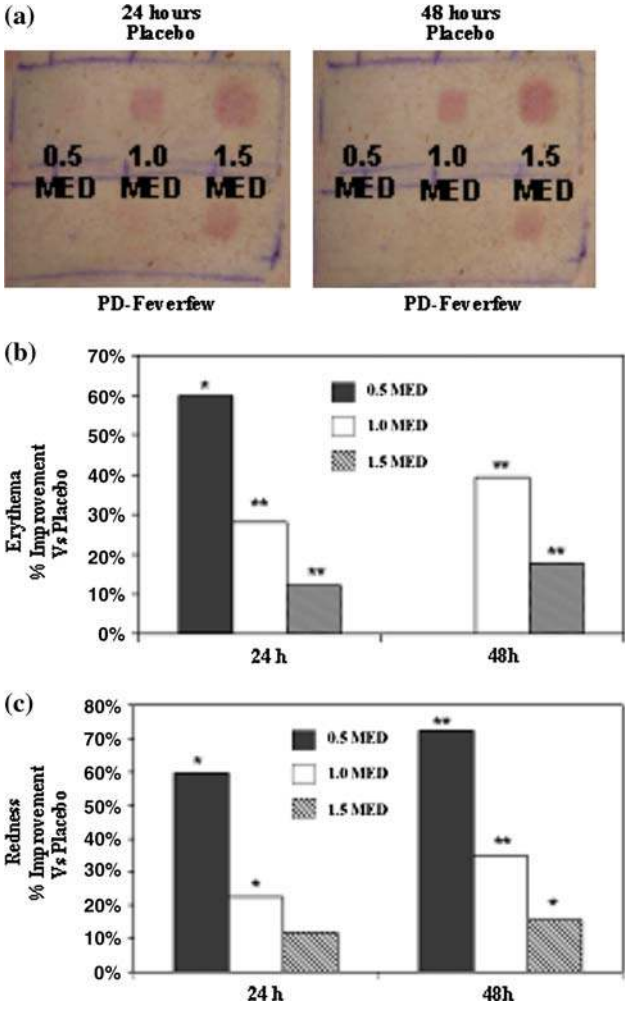

Fig. 5 PD-Feverfew inhibits UV-induced erythema. In a placebo-controlled, randomized, blinded study of 12 subjects, PD-Feverfew formulation treatment followed by exposure to UVB irradiation at varying minimal erythema doses (MED) decreased UVB-induced erythema. **a** Cross-polarized digital photography showing the reduction of erythema. **b** Clinical grader assessment of erythema 24 and 48 h post-UVB irradiation. **c** Assessment of erythema 24 and 48 h post-UVB irradiation using a chromameter. Values are presented as the percentage of improvement relative to placebo. *$P < 0.05$; **$P < 0.1$

Discussion

To eliminate the risk of skin sensitization from *Parthenolide* in Feverfew, which is reported to cause allergic reactions and contact dermatitis [13], we developed a parthenolide-depleted extract of Feverfew (PD-Feverfew) in the current study. Clincial tolerance testing (repeat insult patch testing) of formulations containing PD-Feverfew in over 1,200 subjects demonstrated that PD-Feverfew does not elicit allergic responses and is free of the sensitizing effects of parthenolide [23] in contrast to whole Feverfew. In addition, standard tests of phototoxicity and photoallergy for PD-Feverfew were uniformly negative. While removing parthenolide from Feverfew eliminated the sensitization potential, parthenolide depletion did not reduce the antioxidant activity of Feverfew. We demonstrated that Feverfew, depleted of parthenolide (PD-Feverfew), protects skin from smoke and UV-induced DNA damage, apoptosis and inflammation via the ability to scavenge free radicals and dismutate superoxide, protects cells from oxidant-induced depletion of endogenous antioxidants and induces repair of free radical damaged DNA. Taken together, PD-Feverfew provides broad antioxidant protection and repair activity to the skin, which is continuously challenged by an increasing hostile environment.

DNA damage by UVB irradiation results from photochemical reactions consequent to direct absorption of photons by DNA bases. The UV-induced DNA lesions that have been studied in most detail are the cyclobutane pyrimidine dimer (CPD) and the 6-4 pyrimidine-pyrimidone photoproduct (6-4PP) at adjacent pyrimidines [29]. Our in vivo studies on UV-irradiated microswine showed that PD-Feverfew had a pronounced inhibitory effect on the generation of CPDs (Fig. 4a, b), reducing the formation of CPDs by up to 70%. Several lines of evidence suggest that the reduced DNA damage resulted from the radical scavenging

activity of PD-Feverfew. Nuclear DNA strand breaks are readily produced by incubation of keratinocytes with hydrogen peroxide [2] and hydroxyl radicals can be generated from hydrogen peroxide through Fe^{2+}-mediated Fenton-type reactions [44]. Our study demonstrated that PD-Feverfew scavenged both hydroxyl and ferric radicals (Table 1), while pretreatment with PD-Feverfew abolished the UV-induced release of hydrogen peroxide from normal human keratinocytes (Fig. 2a). In addition, PD-Feverfew reduced the hydrogen peroxide-induced formation of ROS in keratinocytes (Fig. 1c). Furthermore, pretreatment with PD-Feverfew reduced the intrinsic metabolic oxidative stress as shown by the decrease in peroxide levels to below basal (unirradiated control) levels. Thus, it can be concluded that the antioxidant properties of PD-Feverfew can block the cascade of events taking place between UV irradiation and DNA damage. PD-Feverfew treatment resulted in an increase in repair of DNA damaged by UV and chemotherapeutic and photodynamic agents. Thus, PD-Feverfew can provide a unique protection to the skin challenged by oxidative stress, namely protection from ROS and induction of repair pathways in the skin.

The response to chronic UVB irradiation in vivo demonstrated a protective effect of PD-Feverfew on mouse epidermal hyperplasia. A 10 week exposure to low-dose UVB induced a twofold increase in epidermal thickness, which was reduced to 50% by topical PD-Feverfew (Fig. 3). The pattern of epidermal changes were very similar to those observed when green tea polyphenols (0.2%, w/v) were administered orally as a sole source of drinking water [47] or when lutein/zeaxanthin were supplied as a dietary admix [12]. Likewise, PD-Feverfew blocked the UV-induced release of the pro-inflammatory mediator IL-1α from reconstituted human epidermis (Fig. 2b). Cytokine release is one of the myriad early responses to external aggressors, leading, if unchecked, to potential inflammation. Consistent with the mechanism of action of antioxidants, PD-Feverfew was less effective in reducing UV-induced release of the pro-inflammatory mediators when applied only after UV exposure (Fig. 2c). Antioxidants have a greater effectiveness when present prior to, or during, external aggression than when administered after the insult. Indeed, while both pre- and post-treatment of murine keratinocytes with Tocopherol reduced UV-induced PGE_2 release, post-treatment was only about half as effective as pretreatment [51]. In addition to the use of UVB irradiation as the inducer of oxidative stress, we exposed keratinocytes and fibroblasts to cigarette smoke, which is itself rich in free radicals but has also been shown to promote the production of ROS through recruitment and activation of phagocytes in, for example, the lung [39]. Our results showing inhibition of ROS formation, reversible loss of intracellular thiols and a general cytotoxicity are in agreement with published studies [9] and many others reviewed in [46]. PD-Feverfew pretreatment maintained the oxidant–antioxidant balance and thereby allowed the fibroblast thiol content to remain at levels comparable to controls unexposed to cigarette smoke (Fig. 1a). By sparing endogenous antioxidants, PD-Feverfew-treated cells would be better able to resist damage from subsequent external aggression preventing oxidative stress from overwhelming the cellular defenses.

The skin is continuously exposed to numerous forms of external aggression that can alter the oxidative balance and result in cellular damage. Oxidative stress is believed to be a primary factor leading to photoaging and potentially skin cancer [6, 40]. While supplementation of skin with antioxidants putatively can protect skin from oxidative stress, many antioxidants used topically may not be as effective a free radical scavenger as PD-Feverfew. Indeed, antioxidants such as Vitamin C protect against some forms of free radicals, but are marginally effective against other free radicals such as hydroxyl radicals (Table 1) and can undergo degradation when exposed to air [26]. We found that a parthenolide-depleted extract of Feverfew, which was free of sensitization potential, had free radical scavenging activity against a wide range of free radical ions and with greater activity than vitamin C. Furthermore, PD-Feverfew protected skin cells from UV and smoke models of external aggression, reducing free radical damage as shown by maintained cell viability. Through the ability to scavenge free radicals, preserve endogenous antioxidant levels, reduce DNA damage and induce DNA repair of damaged DNA, parthenolide-depleted extract of PD-Feverfew may protect the skin from the numerous external aggressions encountered daily by the skin and reduce the damage to the oxidatively challenged skin.

Acknowledgments We would like to acknowledge Devon Grote, Dara Miller and Judy Pinto for their technical contributions, and Claude Saliou for the helpful discussions on antioxidants. We would like to thank Dr. Lynne B. Harrison for guidance and discussion on the clinical erythema studies.

References

1. Afaq F, Mukhtar H (2002) Photochemoprevention by botanical antioxidants. Skin Pharmacol Appl Skin Physiol 15:297–306
2. Armeni T, Battino M, Stronati A, Pugnaloni A, Tomassini G, Rosi G et al (2001) Total antioxidant capacity and nuclear DNA damage in keratinocytes after exposure to H_2O_2. Biol Chem 382:1697–1705
3. Assefa Z, Van Laethem A, Garmyn M, Agostinis P (2005) Ultraviolet radiation-induced apoptosis in keratinocytes: on the role of cytosolic factors. Biochim Biophys Acta 1755:90–106

4. Aubin F (2003) Mechanisms involved in ultraviolet light-induced mmunosuppression. Eur J Dermatol 13:515–523
5. Babulak S, Rhein LD, Scala DD, Simion AF, Grove GL (1986) Quantitation of erythema in a soap chamber test using the Minolta chroma (reflectance) meter: comparison of instrumental results with visual assessments. J Soc Cosmet Chem 37:475–479
6. Bickers DR, Athar M (2006) Oxidative stress in the pathogenesis of skin disease. J Invest Dermatol 126:2565–2575
7. Bilimoria MH, Ecobichon DJ (1992) Protective antioxidant mechanisms in rat and guinea pig tissues challenged by acute exposure to cigarette smoke. Toxicology 72:131–144
8. Bombardelli E, Morazzoni P (2001) *Tanacetum parthenium* extract and method of obtaining same. United States Patent US6,224,875 B1
9. Carnevali S, Petruzzelli S, Longoni B, Vanacore R, Barale R, Cipollini M et al (2003) Cigarette smoke extract induces oxidative stress and apoptosis in human lung fibroblasts. Am J Physiol 284:L955–L963
10. D'Errico M, Lemma T, Calcagnile A, Proietti De Santis L, Dogliotti E (2007) Cell type and DNA damage-specific response of human skin cells to environmental agents. Mutat Res 614:37–47
11. El Hindi T, Ehlers G, Demchuk M, Pfitzner I (2004) Determination of the antioxidant capacity of an antioxidant combination using the fluoroscan assay in vitro and visualization of its effects using histological methods. Arch Dermatol Res 296:258–264
12. Gonzalez S, Astner S, An W, Goukassian D, Pathak MA (2003) Dietary lutein/zeaxanthin decreases ultraviolet B-induced epidermal hyperproliferation and acute inflammation in hairless mice. J Invest Dermatol 121:399–405
13. Groenewegen WA, Knight DW, Heptinstall S (1992) Progress in the medicinal chemistry of the herb feverfew. Prog Med Chem 29:217–238
14. Haratake A, Uchida Y, Schmuth M, Tanno O, Yasuda R, Epstein JH et al (1997) UVB-induced alterations in permeability barrier function: roles for epidermal hyperproliferation and thymocyte-mediated response. J Invest Dermatol 108:769–775
15. Hausen BM, Osmundsen PE (1983) Contact allergy to parthenolide in *Tanacetum parthenium* (L.) Schulz-Bip. (feverfew, Asteraceae) and cross-reactions to related sesquiterpene lactone containing Compositae species. Acta Derm Venereol 63:308–314
16. Heptinstall S (1988) Feverfew—an ancient remedy for modern times? J R Soc Med 81:373–374
17. Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. Nature 411:366–374
18. Hu HL, Forsey RJ, Blades TJ, Barratt ME, Parmar P, Powell JR (2000) Antioxidants may contribute in the fight against ageing: an in vitro model. Mech Ageing Dev 121:217–230
19. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Deemer EK (2002) Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated beta-cyclodextrin as the solubility enhancer. J Agric Food Chem 50:1815–1821
20. Jain NK, Kulkarni SK (1999) Antinociceptive and anti-inflammatory effects of Tanacetum parthenium L. extract in mice and rats. J Ethnopharmacol 68:251–259
21. Kligman LH (1989) The ultraviolet-irradiated hairless mouse: a model for photoaging. J Am Acad Dermatol 21:623–631
22. Kohen R, Gati I (2000) Skin low molecular weight antioxidants and their role in aging and in oxidative stress. Toxicology 148:149–157
23. Kurtz E, Walczak VR (2005) Clinical safety review of topical emollient formulations containing parthenolide-free-extract of Feverfew. J Am Acad Dermatol 52:P87
24. Kwok BH, Koh B, Ndubuisi MI, Elofsson M, Crews CM (2001) The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IkappaB kinase. Chem Biol 8:759–766
25. MacNee W, Bridgeman MM, Marsden M, Drost E, Lannan S, Selby C et al (1991) The effects of *N*-acetylcysteine and glutathione on smoke-induced changes in lung phagocytes and epithelial cells. Am J Med 91:60S–66S
26. Maia AM, Baby AR, Pinto CA, Yasaka WJ, Suenaga E, Kaneko TM et al (2006) Influence of sodium metabisulfite and glutathione on the stability of vitamin C in O/W emulsion and extemporaneous aqueous gel. Int J Pharm 322:130–135
27. Marks R (1995) An overview of skin cancers. Incidence and causation. Cancer 75:607–612
28. Muller T, Gebel S (1994) Heme oxygenase expression in Swiss 3T3 cells following exposure to aqueous cigarette smoke fractions. Carcinogenesis 15:67–72
29. Nakajima S, Lan L, Kanno S, Takao M, Yamamoto K, Eker AP et al (2004) UV light-induced DNA damage and tolerance for the survival of nucleotide excision repair-deficient human cells. J Biol Chem 279:46674–46677
30. Nguyen H, Finkelstein E, Reznick A, Cross C, van der Vliet A (2001) Cigarette smoke impairs neutrophil respiratory burst activation by aldehyde-induced thiol modifications. Toxicology 160:207–217
31. Nishi J, Ogura R, Sugiyama M, Hidaka T, Kohno M (1991) Involvement of active oxygen in lipid peroxide radical reaction of epidermal homogenate following ultraviolet light exposure. J Invest Dermatol 97:115–119
32. Ogura R, Sugiyama M, Nishi J, Haramaki N (1991) Mechanism of lipid radical formation following exposure of epidermal homogenate to ultraviolet light. J Invest Dermatol 97:1044–1047
33. Ou B, Hampsch-Woodill M, Prior RL (2001) Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agric Food Chem 49:4619–4626
34. Pathak MA, Stratton K (1968) Free radicals in human skin before and after exposure to light. Arch Biochem Biophys 123:468–476
35. Paulsen E, Christensen LP, Andersen KE (2007) Compositae dermatitis from airborne parthenolide. Br J Dermatol 156:510–515
36. Placzek M, Gaube S, Kerkmann U, Gilbertz KP, Herzinger T, Haen E et al (2005) Ultraviolet B-induced DNA damage in human epidermis is modified by the antioxidants ascorbic acid and D-alpha-tocopherol. J Invest Dermatol 124:304–307
37. Rahman I, MacNee W (1999) Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. Am J Physiol 277:L1067–L1088
38. Raitio A, Kontinen J, Rasi M, Bloigu R, Roning J, Oikarinen A (2004) Comparison of clinical and computerized image analyses in the assessment of skin ageing in smokers and non-smokers. Acta Dermatol Venereol 84:422–427
39. Reznick AZ, Cross CE, Hu ML, Suzuki YJ, Khwaja S, Safadi A et al (1992) Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. Biochem J 286:607–611
40. Rittie L, Fisher GJ (2002) UV-light-induced signal cascades and skin aging. Ageing Res Rev 1:705–720
41. Rusznak C, Mills PR, Devalia JL, Sapsford RJ, Davies RJ, Lozewicz S (2000) Effect of cigarette smoke on the permeability and IL-1beta and sICAM-1 release from cultured human bronchial epithelial cells of never-smokers, smokers, and patients with chronic obstructive pulmonary disease. Am J Respir Cell Mol Biol 23:530–536
42. Schwarz T (1988) [The significance of epidermal cytokines in UV-induced immune suppression]. Der Hautarzt; Zeitschrift fur Dermatologie, Venerologie, und verwandte Gebiete 39:642–646
43. Sterenborg HJ, de Gruijl FR, van der Leun JC (1986) UV-induced epidermal hyperplasia in hairless mice. Photodermatol 3:206–214
44. Stewart MS, Cameron GS, Pence BC (1996) Antioxidant nutrients protect against UVB-induced oxidative damage to DNA of mouse keratinocytes in culture. J Invest Dermatol 106:1086–1089

45. Tanaka H, Ono Y, Nakata S, Shintani Y, Sakakibara N, Morita A (2007) Tobacco smoke extract induces premature skin aging in mouse. J Dermatol Sci 46:69–71
46. van der Vaart H, Postma DS, Timens W, ten Hacken NH (2004) Acute effects of cigarette smoke on inflammation and oxidative stress: a review. Thorax 59:713–721
47. Vayalil PK, Mittal A, Hara Y, Elmets CA, Katiyar SK (2004) Green tea polyphenols prevent ultraviolet light-induced oxidative damage and matrix metalloproteinases expression in mouse skin. J Invest Dermatol 122:1480–1487
48. Vink AA, Roza L (2001) Biological consequences of cyclobutane pyrimidine dimers. J Photochem Photobiol 65:101–104
49. Wang H, Kochevar IE (2005) Involvement of UVB-induced reactive oxygen species in TGF-beta biosynthesis and activation in keratinocytes. Free Radic Biol Med 38:890–897
50. Wang H, Liu X, Umino T, Skold CM, Zhu Y, Kohyama T et al (2001) Cigarette smoke inhibits human bronchial epithelial cell repair processes. Am J Respir Cell Mol Biol 25:772–779
51. Yoshida E, Watanabe T, Takata J, Yamazaki A, Karube Y, Kobayashi S (2006) J Invest Dermatol 126:1633–1640