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RESEARCH ARTICLE

Multiple Cytokine Inhibition through Specific Procyanidin – Enriched Plant Extracts: Implications for the Treatment of Psoriasis, Eczema and Dermatitis

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ABSTRACT

Psoriasis, Eczema and Dermatitis (PED) disorders involve abnormally rapid, excessive epidermal cell growth, often followed by secondary infections. Over 20 cytokines, small protein molecules, are thought to act as growth factors. As none of the currently available topical treatments targets all the cytokines implicated in PED, their efficacy is extremely limited, while the systemic administration of immuno-modulating or anti-mitotic drugs has a very poor benefit/risk ratio. Our objective was to identify all the cytokines responsible for PED and to neutralize them using semi-specific procyanidin fractions of plant tannins *in vitro*. 29 purified cytokines considered to be very probably implicated in PED, were applied, either singly or in association, on human epidermal cell cultures to measure their effects. The cytokine association (VB-cytokines) inducing maximum growth was pre-incubated with individual plant procyanidins (PCDs), and the most active PCD association, inducing over 90% VB-cytokine neutralization, was identified. Among 29 active cytokines, we identified 11 cytokines operating in synergy to stimulate excessive, uncontrolled epidermal cell growth. 12 among 134 PCDs, having strong affinities for those proteins, proved capable of blocking all VB-cytokines and normalizing keratinocyte growth. Using specific plant PCDs to inactivate all PED-involved cytokines is a novel and safe scientific approach to treat PED.

Keywords: Cytokine. Dermatitis. Eczema. Psoriasis. Tannins.

INTRODUCTION

Affecting 2-15% of the population, Psoriasis, Eczema, and Dermatitis (PED) are common immune-based inflammatory skin disorders, related to excessive and abnormal cell growth in certain areas of the skin (Fridman et al. 2011). Psoriatic plaques, red and scaly patches on the skin surface generally caused by Psoriasis, are inflammation areas caused by excessive cell growth. Frequently occurring on the elbows and knees, but also on the scalp, hand palms, feet soles, and genitals, those plaques may become infected due to the weakening of the skin tissue (Roberson and Bowcock 2010). In contrast to Psoriasis, Eczema is more likely to be found on the flexor aspect of joints. Eczema lesions are skin rashes characterized by symptoms which include redness, swelling, itching and dryness, sloughing, flaking, blistering, cracking, oozing, or bleeding (Biagini-Myers and Khurana-Hershey 2010). Dermatitis is also linked to anomalous, excessive epidermal cell production, with weakening of the affected areas and possible secondary infections (Brook 2002). The skin becomes red, flaky, very itchy, thick and dry, and may develop bacteria-infected papules (Boguniewicz and Leung 2010). Seemingly distinct, those conditions share the same basic cellular physiopathology, involving a common factor. While their exact causes have yet to be clearly defined, based on patients' history and genome-wide linkage scans, which have identified multiple loci on chromosomes 1q21, 3q21, 17q25 and 20p12 in such patients (Zeeuwen et al. 2008) (Capon et al. 2012), PED disorders are largely attributed to genetic factors. Auto-immunity, immune-modulation, allergic reaction, stress, and other causes may also be involved, but excessive skin cell growth, in correlation with the presence, at the lesion site, of cytokine-secreting cells (neutrophil granulocytes, macrophages, lymphocytes) (Bernard et al. 2012) (Boguniewicz and Leung 2011) and cytokines (Nograles et al. 2008), can be observed in all PED conditions. Cytokines are small, soluble proteins, synthesized by the immune system, and their role includes communication and regulation of cellular activities and cell growth functions. Some cytokines, e.g.: EGF, KGF (Keratinocyte Growth Factor), tumor necrosis factor-alpha (TNF alpha), IL-6 (interleukin-6), IL-22, IL-1B, IL-2, IL-3, IL-4, IL-5, IL-10, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), granulocyte-macrophage colony stimulating factor (rhGM-CSF),

thrombopoietin, IL-23, platelet derived growth factor (PDGF), and fibroblast growth factor (FGF), are thought to act as key growth factors to stimulate cell growth, while some other cytokines, such as macrophage migration inhibitory factor and T-helper 1 (Th1) cytokine interferon-gamma (IFN-γ), are supposed to block cell growth (Ilkovitch 2011) (Nestle et al. 2009). The growth factor-like cytokines are apparently present in very high concentrations in the PED lesions, inordinately promoting cell growth. As a result, the epidermis becomes loosely attached, and prone to microbial contamination, leading to the appearance of symptoms typical of PED. The best treatment solution resides in stopping excessive cell growth by normalizing topical cytokine activity. Suppressing the cytokine-secreting, immune-modulating cells at the lesion site is one way to obtain cell growth regulation. Unfortunately, a medicine capable of blocking the functions of all those immune cells selectively and topically has not been conceived yet. And even if a mechanism were found to block these cells locally, newly synthesized immune cells would keep on circulating and infiltrating into the lesion, limiting the treatment's efficacy. Alternatively, cytokine functions could be blocked by using oral or intravenous preparations. Unfortunately, the cytokines involved are of multiple types, whereas all currently available treatments are very specific, each neutralizing solely one or two molecules, so that significant therapeutic efficacy remains out of reach (Mease 2006), for instance new drugs like adalimumab, infliximab and etarnercept block only TNF-α; kineret blocks IL-1; protopic inhibits IL-2; efalizumab antibodies only bind LFA-1 antigen; and fusion protein alefacept only blocks CD2 receptors on T-cells (Laws and Young 2012). Moreover, the systemic activity of these drugs induces multiple, severe side effects (Roé et al. 2008). Another therapeutic approach would be to employ a topical multiple cytokine inhibitor capable of neutralizing all the cell growth-stimulating cytokines at once, so as to normalize the PED accelerated keratinocyte growth rate without altering systemic immune functions. But the cytokines involved in PED have not all been discovered yet, and the scientific information available is too highly variable to target anti-cytokine research at a defined group of cytokines. Therefore, the aim of this *in vitro* research was to identify all the specific cytokines responsible for the accelerated cell growth in PED lesions and seek means to

neutralize only those particular cytokines, as a safe treatment for PED. Considering that all cytokines and growth factors are proteins, we postulated that they could be neutralized by specific tannins. Tannins are abundant in the plant kingdom and are known for their affinity to bind with a wide range of protein molecules. The procyanidin (PCD) fraction of tannins contains big phenolic compounds with multiple-structure units and may have selective protein-binding properties (Le Bourvellec and Renard 2012). This multiple structure of tannins confers them the capability to form strong hydrogen bonds with various cytokines. *In vitro* methods were employed to identify and ultimately neutralize these cytokines as new therapeutic approach for PED.

MATERIALS AND METHODS

In Vitro Model of Human Skin Cultures

Normal human keratinocytes, in the form of a multi-layered, reconstituted epidermis presenting a histological structure identical to *in vivo* human skin, were used to mimic a PED skin model *in vitro*. Fresh epidermis cultures were purchased from EPISKIN-SNC (Lyon, France). Each epidermal unit consisted of an organotypic culture made of adult human keratinocytes, becoming stratified epidermis after 10-14 days of culture, with 12-15 cell layers at maturity. Cells were prepared from scrapings of normal human skin in modified MCDB-153 cell culture medium (Sigma-Aldrich, France), dissociated enzymatically and seeded onto a collagen (type I) matrix-coated polycarbonate filter capable of absorbing culture medium. Each filter was placed on a sterile sponge, kept in a 6-well tissue culture plate. Only the sponge was immersed in culture medium (3ml MCDB-153 with 10% fetal calf serum) so as to keep the epidermis in an air (outer) to liquid (lower) position, like human skin *in vivo*. Cultures were incubated at 37°C in a 5% CO_2 atmosphere. Under normal conditions (replacing culture medium every 5-6 days), the epidermis is composed of 4-5 cell layers after 1 week of culture, 7-8 after 2 weeks, and 8-12 after 4 weeks. Addition of non-cytotoxic concentrations of growth factors or cytokines to the culture medium increases the speed of keratinocyte growth, resulting in uncontrolled cell proliferation. Excessive cell growth reduces the amount of tissue culture medium and nutrition available, causing keratinocyte sloughing and cell death, similarly to PED skin lesions *in vivo*. Cell growth can be

quantified to evaluate the effect produced by the test products.

Plant Extract PCD Preparation

Procyanidin-rich plant extracts were prepared from 222 tannin-rich plants using methods described by B. Giner-Chavez et al. (1999). Briefly, the primary tannin-rich plant extracts were obtained with an aqueous organic solvent (70% acetone and 30% water). Those extracts were then successively passed through Sephadex LH-20 columns by progressively increasing the volume of methanol (60 x 88.5 cm), and the intended fractions were eluted to produce a dry solid, which was identified by mass spectrometry. The extracts mainly contained procyanidin (epicatechin–catechin) B1, B2, B3 and C1 fractions, between 60% and 80% depending on the plant part used. The concentrated plant extract was then dried by atomization to obtain a PCD-rich plant powder which was stored at 4°C for later use. In preliminary experiments, 134 PCD extracts were selected based on their lack of cytotoxicity, and their cytokine-binding properties. After initial screening, only 32 extracts, showing some anti-cytokine activity, were retained for further evaluation. The key plant PCDs tested were *E. purpurea* (aerial parts), *Mimosa tenuiflora* (bark), *Aesculus hippocastanum* (aerial parts), *Salvia officinalis* (flowering aerial parts), *Alchemilla vulgaris* (aerial parts), *Centella asiatica* (leaves), *Camellia sinensis* (aerial parts), *Acacia catechu* (bark gum), *Vitis vinifera* (leaves), *Prunella vulgaris* (leaves), *Tanacetum parthenium* (aerial parts), *Calendula officinalis* (flowering aerial parts), Oak bark (*Quercus alba*, *Quercus rober*), and fruit extracts of *Sambucus nigra*, *Vaccinium myrtillus*, and *Vaccinium macrocarpon*. For all experiments, PCD-enriched plant extracts were tested at a concentration of 10 mg/ml when used singly, or 5 mg/ml when used in any association.

Cytokine Selection

All cytokines cited in the literature for direct or indirect influence on epidermal cell growth were screened. 29 purified, recombinant test cytokines with known biological activity and insignificant endotoxin levels were purchased from Peprotech (France) and tested: **EGF** (Epithelial Growth Factor also called Insulin-like Growth Factor-II, Somatamedin A); **PDGF-AA** (Glioma-derived growth factor - GDGF, Osteosarcoma-derived Growth Factor - ODGF); **TGF-α** (Transforming Growth

Factor-α, Sacroma growth factor, TGF-type I, ETGF); **TGF-β** (Transforming Growth Factor-beta1, Differentiation inhibiting factor, Cartilage-inducing factor); **TNF-α** (Tumor Necrosis Factor, TNFSF2, Cachectin, Differentiation-inducing factor-DIF, Necrosin, Cytotoxin); **TNF-β** (Tumor Necrosis Factor-beta, TNFSF1, Lymphotoxin-alpha); **FGF-21** (Fibroblast Growth Factor-23); **KGF/FGF-7** (Keratinocyte Growth Factor, Fibroblast Growth Factor-7, HBGF-7); **M-CSF** (Macrophage Colony Stimulating Factor, CSF-1, MGI-IM); **GM-CSF** (Granulocyte/Macrophage Colony-Stimulating Factor, CSF-2, MGI-1GM, pluripoietin-α); **HB-EGF** (Heparin Binding EGF-like growth factor, HBEGF, Diphteria toxin receptor, DTR); **IL-1α** (Hematopoietin-1, Lymphocyte-activating factor-LAF, Endogenous Pyrogen-EP, Leukocyte Endogenous Mediator-LEM), Mononuclear Cell Factor-MCF; **IL-1β** (Catabolin, Lymphocyte-activating factor-LAF, Endogenous Pyrogen-EP, Leukocyte Endogenous Mediator-LEM), **MCF** (Mononuclear Cell Factor); **IL-1β** Interlulin - 1 Beta, Catabolin, Lymphocyte-activating factor-LAF, Endogenous Pyrogen-EP, Leukocyte Endogenous Mediator-LEM, Mononuclear Cell Factor-MCF); **IL-2** (T-cell growth factor-TCGF, Aldesleukin); **IL-3** (MCGF-Mast cell growth factor, Multi-CSF, HCGF, P-cell stimulation factor); **IL-4** (BCGF, BCDF, B cell stimulating factor-BSF-1); **IL-6** (26 kDa protein, IFN-β2, B cell differentiation factor-BCDF, BSF-2, HPGF, HSF, MGI-2); **IL-10** (B-TCGF, CSIF, TGIF); **IL-11** (AGIF-Adipogenesis inhibitory factor); **IL-12** (NKSF, CTL maturation factor-TCMF, Cytotoxic lymphocyte maturation factor-CLMF, TSF); **IL-13** (NC300-Human, P600-Murine); **IL-15** (IL-T); **IL-16** (LCF-Lymphocyte Chemoattractant Factor); **IL-17D** (IL-27); IL-17F; IL-19 (Melanoma differentiation association like protein); **IL-22** (IL-TIF); **FGF-23** (Fibroblast Growth Factor-23); and **SCF** (Stem Cell Factor, c-Kit Ligand, Mast Cell Growth Factor-MGF, Steel Factor). The non-cytotoxic yet biologically active concentration used for each cytokine is indicated in the result tables.

Test Product Exposure

Only young, exponentially growing epidermis cultures, presenting 3-4 cell layers (3-5 days old) were used for the experiments. At the start of each experiment, standard growth medium was replaced by fresh, serum- and cytokine-free medium, under sterile conditions. 50 µl of test product containing appropriate

concentrations of the test substance were then added to the culture medium, and cells were incubated at 37°C in 5% CO_2 atmosphere for a period of 4 extra days to quantify cell growth. Minimum 3 cultures were used to test each concentration. Untreated cells served as negative controls. During preliminary experiments, cells were first exposed to all plant extracts, cytokines, and PCDs, to determine the maximum non-cytotoxic concentration of each test product to be used for further experiments. Cell growth was determined using cell viability MMT test and histological analysis.

Cell Viability Measurements

To quantify live cells, the polycarbonate filter containing cultured epidermis was removed and cut vertically into equal halves. One half was stained with MTT stain to check cell viability as described by Osborne and Perkins (1994). Live cells stain red due to mitochondrial dehydrogenase activity. Red color intensity was evaluated by introducing 100 µl of MTT-stained cell culture solution into 96-well tissue culture microplates and by measuring the optical density (OD) at 650 nm (Dynatec MR 400). The mean OD of untreated cultures served as control reference to which the mean OD values of treated cultures were then compared. Results were expressed on a scale of 0 (100% cell death) to 3, considering that the OD of control cultures was 1 (corresponding to normal number of layers of live cells).

Histological Cell Growth Analyses

The second half of the epidermis was fixed in formaldehyde and stained with standard Hematoxylin & Eosin (H&E) stain for microscopic examination. In short, at the end of the experimental period, each second epidermis half-section was immediately washed with PBS and fixed in buffered 4% paraformaldehyde overnight. Samples were then embedded in a paraffin cassette, and the cassettes were transferred into 70% ethanol and stored at 4°C for later use. For histological evaluation, 5micron-thick vertical sections of epidermis were cut (Microtom, Leica), deparaffinised, rehydrated and stained with H&E stain as described by Li et al (2011). Cell growth in treated cultures was evaluated and compared to the number of cell layers in the negative control cultures (usually around 7-8) which was scored as 1. Scores went from 0 to 3, 0 indicating total cell death, 1 denoting a number of cell layers identical to controls, 2 corresponding to a number of cell layers around 12-14, and 3 indicating excessive

cell growth, with about 15-20 cell layers and cell sloughing. This arbitrary 0-3 scale was chosen because exact quantification is difficult due to cell sloughing and overlap, and it constitutes a useful tool to quantify PED-type of cellular distribution. Scores obtained for cell viability and cell histology at each concentration (n=3 epidermis each) were averaged and mutually compared to evaluate the effects of the test products on cell growth.

Determination of the Cell Growth-stimulating Properties of Cytokines

To determine keratinocyte growth-stimulating properties, exponentially growing, 3-5 days old epidermis was first exposed to each cytokine (n=3) for a 4-day period. Cytokine concentrations ranged between 0.001 and 5 ng/ml, depending on pre-determined biological activity of each cytokine (see result tables for concentrations used). After 4-day incubation, cell growth scores obtained through MTT staining (on a 0-3 scale) and histological examination (on a 0-3 scale) for each epidermal section were added and averaged to a mean cell growth score. The score obtained for negative control (non–cytokine-exposed) epidermis was 1 (normal cell growth), and the percentage of change in cytokine-exposed cells compared to negative controls was calculated. Cytokines accelerating cell growth by over 60% were classified as highly active; between 40 and 60%, as moderately active; between 20 and 39%, as slightly active; and below 20%, as inactive. Highly or moderately active cytokines were then associated with each other to identify the group of cytokines involved in triggering the uncontrolled, excessive growth typical of PED, this combination being named VB-cytokines.

Identification of Cytokine-neutralizing PCDs

The VB-cytokines association was then incubated with each individual PCD for 1 hour at 37°C to allow cytokine-PCD interaction. VB-cytokine activity on cultured epidermal cells was then evaluated as explained above. Unexposed epidermis served as negative control while epidermis exposed only to VB-cytokines served as positive control. Results are expressed as percentage of change induced in cell growth, compared to the positive control. Individual plant PCDs capable of inhibiting VB-cytokines-induced excessive cell growth by more than 10% were then combined to identify the key PCD association capable of maximal normalisation of VB-cytokines-induced cell growth.

Statistical Analyses

Statistical comparisons were made using Student's *t* test. When more than two groups were compared, ANOVA was also employed.

RESULTS

The measured effects of individual cytokines on epidermal cell growth are shown in Table 1.

Only 18 out of 29 cytokines demonstrated cell growth-enhancing properties. EGF, TNF- α , KGF-7, GM-CSF, IL - 1 α, IL-6, and SCF enhanced cell production by over 60% compared to non–cytokine-exposed cells. Moderate cell growth stimulation (between 40 and 60%) was observed with FGF-21, IL-17D, IL-22, and IL-23 while PDGF-AA, TGF- α, TNF - β, M-CSF, HB-EGF, IL-3, and IL-10 were less active as cell proliferation was only increased by 20-40%. All results were statistically significant compared to cell growth in the negative control ($p<0.005$). These cytokines were further evaluated in association with each other to identify the specific cytokine combination most highly responsible for PED cell proliferation. All other cytokines were considered inactive as their cell growth-amplifying effects at biologically active concentrations were below 20%, and statistically not significant ($p>0.05$) compared to negative controls. These results prove that not all but only a few cytokines (11/29) cause accrued epidermal cell multiplication; however the increase in cell growth observed *in vitro* is relatively modest compared to cell growth in PED lesions *in vivo*.

Effect of the Association of Moderately and Highly Active Cytokines on Cell Growth

As shown in Table 2, results indicate that although all the highly or moderately active cytokines conserve their cell growth-enhancing properties, the effects are not additive. EGF + TNFα + FGF-21 + KGF-7, + GM-CSF + IL-1 α + IL-6 + IL-17D + IL-22 + FGF-23 + SCF, all identified as either moderately or highly active cytokines, act in a synergistic manner to accelerate epidermal cell multiplication. Different associations of these cytokines eventually promoted cell growth more than their individual activity, but maximum cell growth was induced only when these 11 cytokines were all present together in the culture medium. This shows that each cytokine acts in synergy with the others to amplify keratinocyte growth.

Table 1: Cell growth-promoting properties of individual cytokines at biologically active concentrations between 0.001 and 5 ng/ml in the culture medium.

Sl. No.	Test Cytokine	Conc. Used Ng/Ml	Average Scores of Mtt and Histology for Each Section			Mean Cell Growth score	% Change
			EXP 1	EXP 2	EXP 3		
	CONTROL	0.0	1.05	1.0	0.95	1.0 ±0.05	0.0
1	EGF	0.1	1.65	1.80	1.70	1.72 ±0.08	+ 72.0%
2	PDGF-AA	0.5	1.30	1.35	1.30	1.32 ±0.03	+ 32.0%
3	TGF-α	0.1	1.25	1.35	1.20	1.27 ±0.08	+ 27.0% *
4	TGF - β	0.025	1.05	1.10	1.20	1.12 ±0.08	+ 12.0% *
5	TNF - α	0.025	1.80	1.65	1.70	1.72 ±0.08	+ 72.0%
6	TNF - β	0.025	1.30	1.40	1.40	1.37 ±0.06	+ 37.0%
7	FGF – 21	2.0	1.65	1.50	1.60	1.58 ±0.08	+ 58.0%
8	KGF – 7	5.0	1.75	1.85	1.80	1.80 ±0.05	+ 80.0%
9	M – CSF	1.0	1.45	1.35	1.30	1.37 ±0.08	+ 37.0% *
10	GM – CSF	0.05	1.75	1.75	1.75	1.75 ±0	+ 75.0%
11	HB – EGF	0.05	1.30	1.30	1.35	1.32 ±0.03	+ 32.0%
12	IL - 1 α	0.001	1.80	1.80	1.65	1.75 ±0.09	+ 75.0%
13	IL – 1 β	0.001	1.0	1.0	1.0	1.0 ±0	0.0%
14	IL – 2	0.05	1.0	1.0	0.95	0.98 ±0.03	8.0% *
15	IL – 3	0.05	1.20	1.25	1.15	1.20 ±0.05	+ 20.0% *
16	IL – 4	0.1	0.90	0.95	0.90	0.92 ±0.03	- 8.0% *
17	IL – 6	0.05	1.70	1.70	1.80	1.73 ±0.06	+ 73.0%
18	IL – 10	1.0	1.30	1.25	1.25	1.27 ±0.03	+ 27.0%
19	IL – 11	0.1	1.0	1.0	1.0	1.0 ±0	0.0%
20	IL – 12	0.05	1.0	1.0	1.0	1.0 ±0	0.0%
21	IL – 13	0.05	1.0	1.0	1.0	1.0 ±0	0.0%
22	IL – 15	0.05	1.0	1.0	1.0	1.0 ±0	0.0%
23	IL – 16 121AA	0.05	0.85	1.0	0.95	0.93 ±0.08	- 7.0% *
24	IL – 17 D	0.05	1.55	1.45	1.60	1.53 ±0.08	+ 53.0%
25	IL – 17 F	0.05	1.0	1.0	1.0	1.0 ±0	0.0%
26	IL – 19	0.05	0.95	1.05	1.0	1.0 ±0.05	0.0%
27	IL - 22	0.05	1.65	1.50	1.55	1.57 ±0.08	+ 57.0%
28	FGF - 23	1.0	1.55	1.50	1.55	1.53 ±0.03	+ 53.0%
29	SCF	1.0	1.70	1.80	1.75	1.75 ±0.05	+ 75.0%
30+	OTHERS	0.01-1.0	<1.10	<1.10	< 1.10	<1.10	< 10.0%

ǂ Mean cell growth score represents the average score (with Standard Deviation) of 3 experiments obtained after MMT and histological analyses. % cell growth change indicates % increase or decrease in cell growth compared to the negative, unexposed control cultures. All experiments were conducted in triplicate. All cell growth changes are statistically significant ($p<0.005$) compared to controls, unless indicated by an asterisk *.

Table 2: Cell growth-promoting properties of associations of highly active and moderately active cytokines, at biologically active concentrations between 0.001 and 5 ng/ml in the culture medium.

Sl. No.	Association of Cytokines Tested	Epidermal Growth Score			Mean Growth Score	% Change
C-	NEGATIVE CONTROL	1.05	1.00	0.95	1.00 ±0.05	0.0
1	EGF + TNFα + FGF-21	1.75	1.65	1.80	1.73 ±0.08	+ 73.33
2	EGF + KGF-7, + IL-1 α	2.05	2.10	1.90	2.01 ±0.*1*	+ 101.67
3	EGF + FGF-23 + SCF	2.05	1.85	1.85	1.92 ±0.11	+ 91.67
4	FGF-21 + KGF-7 + GM-CSF	2.30	2.00	2.20	2.17 ±0.15	+ 116.67
5	EGF + IL-6 + IL-17D + IL-22	1.80	1.75	2.0	1.85 ±0.13	+ 85.00
6	EGF + FGF-21 + KGF-7	2.05	2.25	2.30	2.20 ±0.13	+ 120.00
7	EGF + TNFα + FGF-21 + KGF-7 + GM-CSF	2.30	2.40	2.30	2.33 ±0.06	+ 133.33
8	KGF + IL-6 + IL-17D + IL-22	1.90	1.95	1.90	1.92 ±0.03	+ 91.67
9	KGF + FGF-23 + SCF	2.20	2.10	2.35	2.22 ±0.13	+ 121.67
10	KGF-7 + GM-CSF	2.20	2.10	2.10	2.13 ±0.06	+ 113.33
11	IL-6 + IL-17D + IL-22	1.80	1.95	1.70	1.82 ±0.13	+ 81.67
12	EGF + TNFα + FGF-21 + KGF-7, + GM-CSF + IL-1 α + IL-6 + IL-17D + IL-22 + FGF-23 + SCF	2.80	2.65	2.60	2.68 ±0.1	+ 168.33
13	OTHER ASSOCIATIONS	<1.50	<1.50	<1.50	<1.50	< +50

ǂ Mean cell growth score represents the average score (with Standard Deviation) of 3 experiments obtained after MMT and histological analyses. % cell growth change indicates % increase or decrease in cell growth compared to unexposed control cultures. All experiments were conducted in triplicate and all cell growth changes are statistically significant ($p<0.005$) compared to controls.

Table 3: VB-cytokine inhibitory effect of PCDs: either singly or in different associations of active PCDs.

Sl. No.	Test Cytokine And Plant Extract Code	Mean Growth Score	% Change Vs Negative Control	% Change Vs Positive Control
C -	NEGATIVE CONTROLS	1.0	0.0 %	OK
C +	POSITIVE CONTROLS	2.68	+ 168.3%	+168.3
1	AC	2.03	+ 103 %	-38.8
2	SO	2.05	+ 105 %	-37.6
3	VV	1.61	+ 61 %	-63.76
4	QR	2.38	+ 138%	-18.0
5	GB	2.06	+ 106 %	-37.01
6	V SP	1.43	+ 43 %	-74.4
7	SA	1.57	+ 57 %	-66.13
8	MT	2.32	+ 132 %	-21.56
9	EP	2.35	+ 135 %	-19.79
10	TP	2.27	+ 127 %	-24.54
11	CS	1.77	+ 77 %	-54.25
12	SN	1.50	+ 50%	-70.29
13	OTHERS			
ASSOCIATION				
14	AC + VV	1.83	+ 83 %	-50.68
15	AC + MC	1.78	+ 78 %	-53.65
16	AC + EP	1.55	+ 55 %	-67.32
17	AC + CS	1.55	+ 55 %	-67.32
18	VV + GB	1.61	+ 61 %	-63.76
19	MC + EP	1.52	+ 52 %	-69.10
20	MC + CS	1.65	+ 65 %	-61.38
21	EP + CS	1.35	+ 35 %	-79.20
22	VV + V SP	1.18	+ 18 %	-89.30
23	VV + CS	1.25	+ 25 %	-85.15
24	VV + SN	1.30	+ 30 %	-82.17
25	VV + V SP + CS	1.14	+ 14%	-91.68
26	VV + MT + SN	1.38	+ 38%	-77.42
27	SN + V SP + CS	1.22	+ 22%	-86.93

ǂ Cell growth score of unexposed and untreated negative control cultures was considered as 1 (100% cell growth). Positive controls were exposed only to VB-cytokines. Change in cell growth is expressed as % increase (+) or decrease (-) in cell growth mean score compared to the positive VB-cytokine-exposed cultures. All values are means of at least 3 experiments and all values are statistically significant compared to the positive controls ($p<0.005$).
1 = *Acacia catechu*; 2 = *Salvia officinalis*; 3 = *Vitis vinifera*; 4 = *Quercus robur* (Oak bark); 5 = *Ginko biloba*; 6 = *Vaccinium sp*. (fruits); 7 = *Salix alba*; 8 = *Mimosa tenuiflora*; 9 = *Echinacea purpurea*; 10 = *Tanacetum parthenium*; 11 = *Camellia sinensis*; 12 = *Sambucus nigra*. Others = as given in the list of plants tested.

Cytokine-inhibiting Properties of Plant PCDs

VB-cytokines were incubated with a fixed concentration (10 mg/ml singly, or 5 mg/ml each when used in association) of PCDs from more than 130 tannin-rich plants for 1 hour, and epidermal growth was then quantified. As shown in Table 3, in negative controls, without addition of VB-cytokines or PCDs, cell growth was normal (scored as 1) with the epidermis showing 6-7 cell layers. In VB-cytokines positive control group, (VB-cytokines only, no PCDs), the number of epidermal cell layers was 17-19, with inordinate cell sloughing, representing excessive cell growth with mean values 168.3% higher than negative controls. Pre-incubation of the same VB-cytokines with individual plant PCDs showed no statistically significant changes in cell growth for 120 of 132 PCDs, indicating absence of binding between these PCDs and the cytokines. Only 12/132 PCDs decreased the excessive cell growth by 15% to 74% compared to positive controls ($p<0.005$). The highest VB-cytokines inhibition was observed with PCDs obtained from *Vaccinium sp*. (*Vaccinium macrocarpon*, *V. Myrtillus*) and *Sambucus nigra*, since they each inhibited VB-cytokines-induced keratinocyte proliferation by about 70%. PCDs from *Vitis vinifera*, *Salix alba*, and *Camellia sinensis* were also effective, decreasing VB-cytokines-induced cell multiplication by 50-60%. Results obtained with PCD associations indicate that *V. vinifera* or *S. nigra* with *Vaccinium sp*. or *C. sinensis* prove extremely effective in neutralizing VB-cytokines, as mean excessive epidermal growth was reduced by 80-91% compared to positive controls. These results clearly show that PCDs are highly specific with respect to their cytokine binding and that only a few PCDs have the structural configuration to bind with the cytokine proteins.

DISCUSSION

Normal keratinocyte maturation cycle of 28-30 days is accelerated to 4-5 days in PED-affected skin, resulting in poor cell attachment, cell sloughing, secondary infections and persistence of PED lesions (Halprin 1972). PED-type skin diseases are almost uniformly considered to result from changes in the body's immunological functions, some immunological reactions probably causing tissue damage and a localized inflammatory zone on the skin (Guttman-Yassky et al. 2011). When the skin detects a danger signal, a cascade of highly sophisticated interactions between keratinocytes and "sentinel" immune cells

is triggered to maintain skin homeostasis. The inflammation and subsequent cell regeneration are relayed by more than 20 or 30 different types of small communication protein molecules, called cytokines, each playing a specific role in the skin damage repair process. For a number of known or unknown reasons (genetic, environmental, etc.), faulty signals occur during the resolution of this phenomenon and generate a cytokine-mediated vicious circle, promoting chronic inflammation, huge infiltration of immune cells in the dermis and epidermis, production of chemokines and growth factor-like cytokines, altered differentiation of keratinocytes, uncontrolled and excessive cell production, and development of skin lesions classified as psoriasis, eczema or dermatitis, depending on the location and appearance (Guilloteau et al. 2010), although the basic physiopathology of those three conditions remains more or less identical. Different authors discovered the predominance of multiple types of proteins and cytokines acting as growth factors, such as IL-1 (IL-1α and β), IL-2 (IL-4, IL-13, IL-21), IL-4 (IL-4, IL-13), IL-6 (IL-6, OSM, IL-31), IL-10 (IL-19, IL-20, IL-22, IL-24), IL-17 (IL-17A and IL-17F), IFN (IFNα, IFNγ), or TNF (TNFα, TNFβ) family (Caruso et al. 2009), but other cytokines involved in the pathology have not yet been identified. Extreme innate immune response prompting cytokine production, epidermal basal layer cell over-proliferation, epidermal layer disruption, in conjunction with mechanical damage from intense pruritus and desquamation, contribute to the more severe sequelae, including chronic bacterial colonization (*S. aureus*) and penetration of exogenous substances, such as allergens, irritants, microbes, pollutants, and even topical drugs, into the PED lesions. There is, as yet, no treatment which can normalize the body's immune functions to derail the uninterrupted inflammatory trigger, recruitment of immuno-modulating cells in the PED lesion, and excessive production of growth factor proteins, so as to normalize keratocyte growth without severe side effects (Chaudhari et al. 2001). Moreover, modifying the body's immunological mechanisms might stop PED pathogenesis but would equally affect all other immunological functions of the body, which may prove disastrous for the patient's health. Considering the absence of a truly effective treatment for PED and the side effects associated with the immune-modulating or biological

therapies currently employed, products acting only topically may represent the therapy of choice for treating PED. As PED skin is damaged and therefore highly permeable, topical treatment efficacy should be facilitated. If the lesion is smooth or keratinized, scraping the surface just enough to render it permeable should allow PCDs to reach the site of cellular proliferation. Since the basic cause of PED is excessive cell production and certain cytokines act as growth factors to maintain cell proliferation, it was important to identify all cytokines specifically implicated in PED so as to envisage a treatment. As it is practically impossible to control the local functions of immune-modulating cells with a single chemical molecule without affecting vital cellular functions in other parts of the body, we employed plant tannins as natural multiple protein inhibitors to neutralize the growth-promoting cytokines. Currently, only methotrexate is commonly employed to reduce cell growth in PED (Lee et al. 2012). This is an orally-administered anti-cancer drug which stops cell growth in the S-phase of the cell cycle. Unfortunately, none of the anticancer drugs can differentiate between normal and pathological cells, and they equally affect the growth of healthy cells. Consequently, their benefit/risk and cost/risk ratios are extremely poor, not warranting their use to treat a non life-threatening disease like PED. Moreover, such drugs have no effect on cytokines, the main cause of symptomatic manifestations of immunological disorders (Le Bourvellec and Renard 2012). It was therefore essential to target only those cytokines involved in PED cell growth, without affecting the functions of other cytokines which may actually be useful to repair PED lesions. Results of *in vitro* studies have shown that as many as 29 cytokines possess skin cell growth-promoting properties but only 11 of them act synergistically to induce uncontrolled cell proliferation, the basic cause of PED disorders. Tannins and PCDs are very big, highly branched, safe molecules having a strong affinity for proteins. We observed that allowing tannin–cytokine interaction effectively blocks the biological activity of cytokines, but that only a few PCDs have the capacity to bind with the VB-cytokines. We postulated that applying topically a synergistic association of selected cytokine-inhibiting plant PCDs might curb cytokine-induced cell proliferation, and we pursued this research hypothesis to conceive a new therapeutic strategy, focused on neutralizing the cause

of excessive cell growth by targeting only growth-factor-like cytokines rather than using non-specific systemic therapies or interfering with immunological cellular functions. Although further *in vivo* and clinical trials are recommended to either confirm or refute this hypothesis, the specific blockage and inactivation of cytokines responsible for epidermal cell proliferation offers a new alternative for the treatment of PED.

Acknowledgements & Declaration of Interests

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Conflict of Interest statement

The Authors report no conflict of Interest.