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Echinacea purpurea-derived alkylamides exhibit potent anti-inflammatory effects and alleviate clinical symptoms of atopic eczema

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ABSTRACT

Background
Atopic eczema (AE) is a chronic inflammatory and pruritic skin disease. There is still an unmet need for topical anti-inflammatory and anti-pruritic substances exhibiting an excellent safety profile. The endocannabinoid system is known to regulate various aspects of cutaneous barrier and immune functions, thus targeting it may be a valid approach for alleviating the symptoms of AE.
Objective
To assess the putative efficacy of *Echinacea purpurea*-derived alkylamides (Ec. extract) activating cannabinoid (CB)-2 receptors in exerting anti-inflammatory effects and alleviating symptoms of AE.
Methods
In vitro anti-inflammatory efficiency was investigated by monitoring the effects of Ec. extract on poly-(I:C)-induced pro-inflammatory cytokine expression (Q-PCR) and release (ELISA) of HaCaT keratinocytes. Irritancy and sensitization potential (assessed by Human Repeat Insult Patch Test; Clinical trial 1); clinical efficiency in alleviating symptoms of AE (Clinical trial 2) as well as effects on human skin structure and lipid content (Clinical trial 3 followed by transmission electron microscopy and HPTLC) were investigated in randomized double blind clinical trials.
Results
Ec. extract significantly reduced mRNA expression as well as release of poly-(I:C)-induced pro-inflammatory cytokines (IL-6 and IL-8) in keratinocytes. Thus, not surprisingly, the well-tolerated (Clinical trial 1) Ec. extract-based cream reduced local SCORAD statistically significantly, not only compared to baseline, but also compared to the comparator (Clinical trial 2). Of great importance, besides the *in vitro* anti-inflammatory effects, administration of the Ec. extract-based cream also resulted in significantly higher levels of overall epidermal lipids, ceramide EOS (ω-esterified fatty acid + sphingosine sphingoid base), and cholesterol at Day 15 compared to baseline as well as significantly greater numbers of intercellular lipid lamellae in the intercellular space (Clinical trial 3).
Conclusion
The investigated Ec. extract shows great potential in alleviating cutaneous symptoms of AE, and by exerting remarkable anti-inflammatory actions and restoring the epidermal lipid barrier, it will be very likely a well-tolerated, powerful novel ingredient for the adjuvant therapy of AE.

1. Introduction

Atopic eczema (AE), also known as atopic dermatitis, affects between 2 and 10% of adults and 15–30% of children, and is considered to be the most common inflammatory skin condition in childhood [1]. The major clinical symptoms of AE are erythema and pruritus leading to the well-known itch/scratch cycle [2]. The underlying etiology of AE is multifactorial, including a genetic predisposition, deficiencies in the skin of specific polyunsaturated fatty acids (such as linoleic acid), skin barrier defects, and immune dysfunction, where the skin barrier defects are considered central [3,4]. Thus, it is not surprising that ceramide levels in the stratum corneum are decreased in these patients. In a study conducted by Imokawa et al. in the early 1990s, there was a marked reduction in ceramide levels in both lesional and non-lesional skin in AE patients, compared with healthy subjects [5]. Findings from a more recent study suggested that metab-

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olism of sphingomyelin, a precursor to barrier ceramides, is altered in AE, leading to a decrease in ceramide levels. The resulting ceramide deficiency may contribute to a disturbed epidermal barrier with increased transepidermal water loss (TEWL) thus leading to dry skin, another clinical symptom associated with AE [6,7]. Hence, increasing ceramide levels within the epidermal barrier may improve AE symptoms, such as erythema or pruritus.

Currently, emollient treatment is the basic therapy to improve skin barrier function in addition to the use of topical corticosteroids or immune modulators in AE [8,9]. Moreover, plant-extract containing formulations may add to the basic therapy exhibiting additional effects and have shown promise in treating mild AE [10]. However, there is still an unmet need to identify novel therapeutic targets exhibiting "favorable" side-effect profiles.

The endocannabinoid system (ECS) is a multifaceted signaling system regulating a wide variety of physiological functions in the central nervous system as well as in the periphery, including regulation and control of the inflammatory/allergic processes [11]. Since there is a functioning endocannabinoid system (ECS) existing in the skin, targeting it may be a valid approach for alleviating AE symptoms [12]. It seems that the main physiological function of the cutaneous ECS is to constitutively control the proper and well-balanced proliferation, differentiation and survival, as well as immune competence and/or tolerance of skin cells [12].

Indeed, recent data showed that loss of appropriate cannabinoid signaling exacerbated allergic skin inflammation [13], whereas increase of the endocannabinoid tone by inhibiting fatty acid amide hydrolase (FAAH; one of the major endocannabinoid degrading enzymes) prevented Toll-like receptor activation induced inflammatory response of human immortalized keratinocytes via indirect activation of CB_1 and CB_2 cannabinoid receptors, and substantially alleviated skin symptoms of NC/Tnd mice [14]. Collectively, these results strongly argue for that the homeostatic endocannabinoid signaling may be a crucial gate-keeper in controlling cutaneous immune processes. Moreover, several non-psychotropic phytocannabinoids (namely (−)-cannabidiol, (–)-cannabichromene, (–)-cannabidivarin, (–)-cannabigerol, (–)-cannabigerovarin and (–)-Δ^9-tetrahydrocannabivarin) were recently shown to exert promising anti-inflammatory effects in human sebocytes, further arguing for that plant-derived agents influencing certain branches of the complex cannabinoid signaling may have potential in the management of cutaneous inflammations [15,16].

It is well-known that *Echinacea purpurea and E. angustifolia* preparations are widely used to treat upper respiratory infections, and it is thought that many of their benefits are due to their immunomodulatory properties, including effects on cannabinoid receptors [17,18]. One study showed that *Echinacea* spp.-derived alkylamides (i.e. dodeca-*2E*,*4E*,*8Z*,*10Z*-tetranoic acid isobutylamide [A1] and dodeca-*2E*,*4E*-dienoic acid isobutylamide [A2]) bind to CB_2 receptor even more strongly than endogenous cannabinoids [19]. In another study, alkylamides from *Echinacea* extracts were shown to modulate expression of tumor necrosis factor-α (TNF-α) mRNA in human macrophages and monocytes via CB_2 receptor, and inhibition of lipopolysaccharide (LPS)-stimulated TNF-α expression by alkylamides was also demonstrated [20]. Alkylamides derived from *Echinacea* spp. have also been shown to partially inhibit the activity of FAAH [21], indicating that, besides direct CB_2 binding, they can also positively modulate receptor activity by increasing the endocannabinoid tone. Based on these data, *E. purpurea*-derived extracts hold out the promise to exert beneficial (i.e. anti-inflammatory and anti-pruritic) effects in AE.

To prove the benefit of a water-in-oil (W/O) emulsion containing an *E. purpurea* extract for daily skin care of atopic individuals, an *in vitro* study was first conducted which assessed putative anti-inflammatory effects of the *E. purpurea* extract in human keratinocytes. Thereafter, cutaneous tolerability, clinical efficacy and effects on stratum corneum structure and lipid levels of an *E. purpurea* extract-containing W/O-emulsion were investigated in subjects suffering from AE.

2. Materials and methods

2.1. Echinacea purpurea extract (Ec. extract)

Echinacea purpurea is cultured according to Good Agricultural and Collection Practice in the Netherlands, Poland and Ukraine. Following harvest in autumn dried roots are subjected to a quality check and are stored not exceeding March of next year before the roots are extracted. The extract is prepared by supercritical CO_2-extraction of *Echinacea purpurea* roots and alkamide content is analyzed analog to Sun et al. [22]. The method separates the lipophilic components from the remaining plant materials and leaves no solvent residues.

The extract is formulated in a cream of the water-in-oil emulsion type and consists of the following ingredients: Aqua, Decyl Oleate, Isopropyl Myristate, Carthamus Tinctorius Seed Oil, Hexyldecanol, Hexyldecyl Laurate, Glycerin, Polyglyceryl-3 Polyricinoleate, Sorbitan Isostearate, Cera Alba, Citrus Aurantium Dulcis Peel Extract, Zinc Stearate, Benzyl Alcohol, Echinacea Purpurea Root Extract, Magnesium Sulfate, Lecithin, Tocopherol, Ascorbyl Palmitate, Glyceryl Oleate, Glyceryl Stearate, Citric Acid.

2.2. In vitro study

2.2.1. Materials

The Toll-like receptor 3 activator polyinosinic-polycytidylic acid (poly-(I:C)) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly-(I:C) was dissolved in filtered distilled water, whereas the solvent for the *E. purpurea* extract (Ec. extract; provided by Dr. August Wolff GmbH & Co. KG Arzneimittel, Bielefeld, Germany) was dimethyl sulfoxide (DMSO; VWR International Ltd., Debrecen, Hungary).

2.2.2. Cell culturing

Human immortalized HaCaT keratinocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (Life Technologies Hungary Ltd., Budapest, Hungary), and antibiotics (MycoZap™ Plus-CL in 1:500; Lonza, Budapest, Hungary) at 37 °C in a 5% CO_2-containing humidified atmosphere. The medium was changed every other day, and cells were sub-cultured at 70–80% confluence.

2.2.3. RNA isolation, reverse transcription, quantitative "real-time" PCR (Q-PCR)

RNA isolation, DNase treatment, reverse transcription and Q-PCR were performed as described previously [14,15,23]. PCR amplification was performed by using TaqMan® primers and probes (assay ID-s: Hs00985639_m1 for *IL-6* and Hs00174103_m1 for *IL-8*) and the TaqMan® universal PCR master mix protocol (Applied Biosystems). As internal controls, transcripts of *18S RNA* were measured (assay ID: Hs99999901_s1). The expression of transcripts was normalized to those of the housekeeping gene, and results were then normalized to the mRNA expression of the vehicle control (ΔΔCT method).

2.2.4. Determination of cytokine release (ELISA)

Cells were treated as indicated for 3 and 24 h. Supernatants were then collected, and the released amount of IL-6, and IL-8 were determined using OptEIA kits (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's protocol.

2.2.5. Statistical analysis

Data were analyzed and graphs were plotted by using Origin Pro Plus 6.0 software (Microcal, Northampton, MA, USA), using Student's two-tailed two samples *t*-test (paired comparisons).

2.3. Clinical study 1: irritancy and sensitization potential

2.3.1. Study design

This randomized, double-blind, single-center, placebo-controlled clinical study was conducted in healthy subjects in Hamburg, Germany in order to determine cutaneous tolerability of the Ec. extract containing W/O emulsion (WO 3260) by assessing local irritancy and sensitization potential compared to the very well-tolerated and successfully marketed Linola® body lotion (Dr. August Wolff GmbH & Co. KG Arzneimittel) in a human repeat insult patch (HRIP) test performed as described previously [24].

2.3.2. Patient selection

Of the 105 volunteers recruited, 104 were eligible for data analysis. Inclusion criteria were: aged between 18 and 75 years, skin phototype I–IV, and in general good health. Exclusion criteria included: acute cutaneous diseases at the skin test sites, known hypersensitivity to Fixomull® Stretch plasters or to the test products, and application of topical medication in the test area within the month before study start.

Volunteers were Caucasian, aged between 19.9 and 74.2 years (mean of 47.9 years ±13.2) and comprised 89 and 15 females and males, respectively.

2.3.3. Treatment schedule

Induction/irritancy potential phase: test products (i.e. Linola® body lotion and the Ec extract-containing "WO 3260") or placebo were applied 6 times (150 μl per application) to different test sites on the lower back of each volunteer over a 14-day period (48 h, 48 h, 72 h, 48 h, 48 h, 72 h). Challenge/sensitization potential phase (1 week after the induction phase): a single 150 μl application of a test product or placebo was assessed over 48 h.

In both phases, applications were administered with the test patch device FinnChamber® Large (18 mm) and a Fixomull® Stretch plaster was subsequently placed over the test site.

As a result of dropouts and treatment discontinuations within the first 8 days of the study as a result of an erythema score of 1 or higher, 80 of the volunteers were challenged with Linola® body lotion and 85 of the volunteers were challenged with WO 3260.

2.3.4. Safety assessments

Irritancy and sensitization were assessed using a 0–6 point ranking system for erythema. Grading was as follows: 0 = no reaction, 0.5 = slight, patchy erythema, 1 = slight uniform erythema, 2 = moderate, uniform erythema, 3 = strong erythema, 4 = strong erythema, spreading outside of patch, 5 = strong erythema, spreading outside of patch with swelling or vesiculation, 6 = severe reaction with erosion. Any other sign of cutaneous irritancy was also to be recorded. Visual assessments were performed on days 3, 5, 8, 10, 12 and 15 in the induction/irritancy phase and 10 to 30 min and 48 h after patch removal in the challenge phase. The irritancy potential for each volunteer was assessed by the Cumulative Irritation Score (calculated as the sum of the daily scores) during the irritancy phase.

2.3.5. Statistical analysis

For missing data the last observation was carried forward. The products were compared to each other using Friedman's ANOVA and *post-hoc* comparisons (Wilcoxon-Wilcox-Test).

2.4. Clinical study 2: clinical efficacy in patients with AE

2.4.1. Study design

Clinical efficiency was tested in a prospective, randomized, double-blind, clinical study conducted at a test center in Hamburg, Germany over a period of 3 months. It is well-known that comparison with the placebo could result in the preference of the less smelly product by the subjects in a clinical trial. Thus, since due to the presence of the extract, WO 3260 (Linola® Plus Cream, Dr. August Wolff GmbH & Co. KG Arzneimittel, Germany) has a characteristic odor which cannot be masked or mimicked efficiently, it was impossible to assess its efficiency in a placebo-controlled study. Since we wanted to make sure that the smell would not be a discriminator, we selected a clinically efficient [25] comparator product (Imlan® Creme Pur, Birken AG, Germany) also possessing a characteristic odor. Thus, the aim of Clinical study 2 was to compare the effects of WO 3260 with Imlan® Creme Pur in patients suffering from AE. The primary endpoint was the change from baseline in local SCORAD score (sum of scores for erythema, edema/papules, weeping/crusts, excoriation, lichenification, pruritus and dryness) at Day 29, Day 57 and Day 85 in the per-protocol (PP) population.

2.4.2. Patient selection

Overall, 60 male and female patients were enrolled and comprised the safety and intention-to-treat (ITT) populations. Of these patients, 11 were excluded (4 due to the test product and 7 for other reasons) and consequently the PP population comprised 49 patients. Other inclusion criteria were: age at least 10 years, AE with at least two slight eczematous and contralaterally-located subacute or chronic lesions in the test area without any sign of infection as rated by the dermatologist or an experienced physician (local SCORAD at least 4; erythema and pruritus scores at least 1). Exclusion criteria included: active skin disease other than AE at test area, serious health condition potentially affecting the test reaction/assessment, topical medication at the treatment area on the day before study start and/or during the study period, systemic treatment with antiinflammatories, analgesics, immunosuppressive drugs, antihistamines and/or antibacterials on the day before and/or during the study period. Demographic data for the safety/ITT and PP populations are summarized in Table 1. Flow diagram for patient inclusion is summarized in Fig. 1.

2.4.3. Randomization and treatment schedule

Validated software (Randomization in Treatment Arms [RITA]; Evidat®, Sereetz, Germany) was used to assign the two test products to their respective test areas minimizing inter-individual differences in local SCORAD values. A dermatologist or an experienced physician identified 2 comparable and contralateral skin areas (crooks of arms, knee hollow, trunk, wrist or shin) with mild AE lesions. WO 3260 or reference product was applied 2 or 3 times daily. Subjects were instructed to apply the test product within 'area 1′ and the reference product within 'area 2′ on the contralateral side of their body.

Table 1
Demographic features of the study population and the per protocol population (Clinical trial 2).

Population	SP (Enrolled, N = 60)	PP (Valid, N = 49)
Age (mean ± SD [range])	30.2 ± 15.9 (10–85)	32.5 ± 16.4 (10–85)
Gender (N [%])		
female	38 (63.3)	30 (61.2)
male	22 (36.7)	19 (38.8)
Received concomitant diagnosis (N [%])	60 (100)	49 (100)
On concomitant therapies (N [%])	32 (53.3)	26 (53.1)

PP = per protocol; SD = standard deviation; SP = safety population.

2.4.4. Clinical assessment

Efficacy was objectively assessed with local SCORAD (sum of 7 SCORAD categories). Local SCORAD is considered to be one of the best tools for clinical assessment of localized AE [26].

Local SCORAD parameters: Erythema, edema and papules, weeping and crusts, excoriation, lichenification, pruritus on lesions and dryness on the non-lesional areas.

Scale: 0 = None; 1 = Slight; 2 = Moderate; 3 = Strong. The local SCORAD was calculated as sum of grading of the seven parameters mentioned above; thus, the maximum value of the local SCORAD could be "21".

Besides local SCORAD, subjective efficacy was also assessed by asking for feeling dryness, burning, tension, tickling or other unpleasant sensations. Scale: 0 = None; 1 = Slight; 2 = Moderate; 3 = Strong. The subjects also filled in a product acceptance questionnaire with different scores concerning test material traits. Clinical assessments were performed on Days 29, 57, and 85.

2.4.5. Safety assessments

Adverse reactions in the treatment areas were scored for the relevant parameter at scheduled visits. All adverse reactions were documented in study records.

2.4.6. Statistical analysis

Computation of the statistical data was performed with a commercially available statistics program (SAS for Windows; SAS Institute GmbH, Heidelberg, Germany). N, means, standard deviations and 95% confidence limits of all subjects in the population (PP) were calculated for metric scaled data at all assessment times by test product as well as for calculated parameters. A significance level of 0.05 (alpha) was chosen for statistical analysis. Due to the explorative nature of this study, no adjustments for multiplicity were made. To assess whether treatments differed in local SCORAD a repeated measurements ANOVA test on the changes from baseline with the factors of treatment and time and the according interaction was performed. Ad-

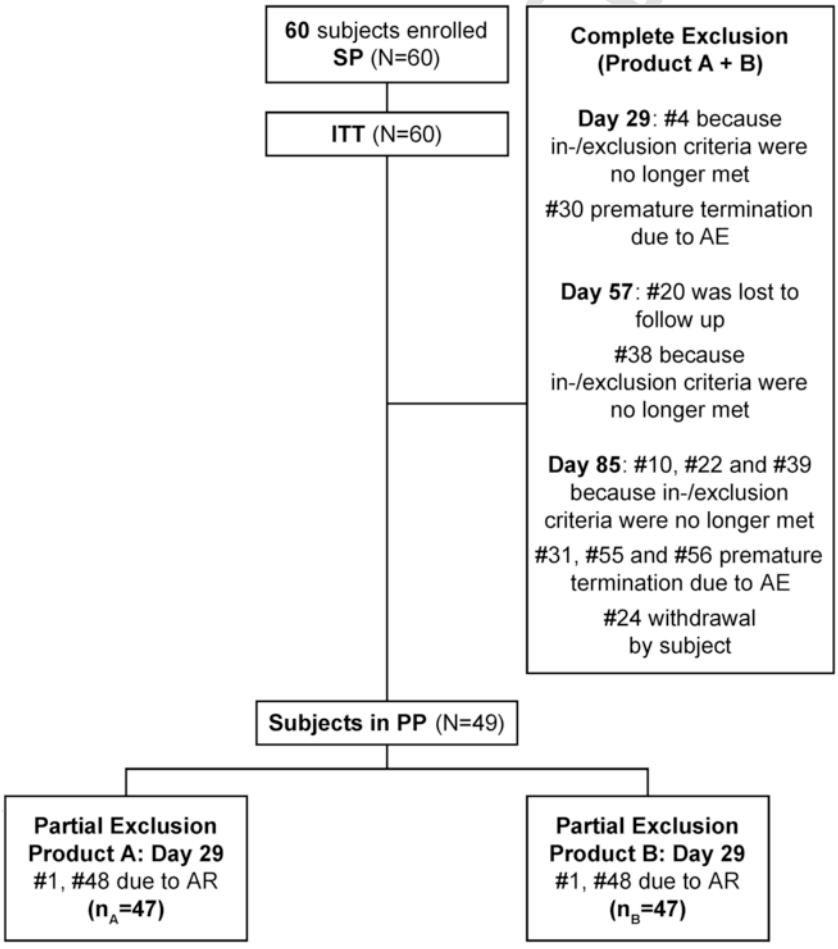

Fig. 1. Flow diagram for patient inclusion (**Clinical trial 2**). **AR:** adverse reaction; **ITT:** intention-to-treat population; **PP:** per protocol population; **SP:** study population.

ditional paired *t*-tests were conducted to compare changes from baseline in local SCORAD at Days 29, 57, and 85 for each treatment and to compare these changes between treatments.

2.5. Clinical study 3: skin barrier function

2.5.1. Study design

This randomized single-center study conducted in Hamburg, Germany assessed the effects of WO 3260 on skin barrier parameters in subjects with AE.

2.5.2. Patient selection

A total of 25 male and female subjects were recruited. Other eligibility criteria included: age between 18 and 70 years, skin phototype I–III, AE with local SCORAD of between 1 and 15.

Overall, 22 were female (88%) and 3 were male (12%). The mean age was 45.4 (±13.9) years. Fifteen subjects were included in the transmission electron microscopy (TEM) and lipid analyses.

2.5.3. Treatment schedule

WO 3260 (approximately 2 mg/cm^2) was applied twice a day to a designated test area on the forearm of subjects over a 14-day period.

2.5.4. Skin barrier assessments

Skin barrier assessments were carried out on Day 15. TEM was used to assess change in ultrastructure and amount of lipid lamellae in stratum corneum (SC). Components of the intercellular lipid lamellae (ceramide EOS, ceramide NP, ceramide NH, cholesterol, free fatty acids) were assessed by a lipid analysis (high performance thin layer chromatography HPTLC).

Both analyses utilized Lipbarvis®, a non-invasive sampling technique to harvest stratum corneum samples [27]. Therefore, a drop of the glue was applied to the carrier, which was then placed onto the skin surface. After polymerization, the carrier with the adhering corneocytes was gently removed from the skin surface. This procedure was repeated a second time at the same location. This sample was then used for TEM and for lipid analysis.

A part of the sample was prepared for subsequent TEM analysis according to Dähnhardt-Pfeiffer et al. [28]. The TEM investigation with a transmission electron microscope CM10 (FEI, Eindhoven, The Netherlands) shows the intercellular lipid lamellae in the intercellular space in the SC samples. In the subsequent morphometric analysis the length of the intercellular lipid lamellae (ICLL) in the intercellular space (ICS) are set into a relationship to the surface area of the intercellular space and calculated with respect to a reference value of 1000 nm^2 (nICLL). This quotient allows a comparison of lipid lamellae between treated and untreated areas of the skin.

2.5.5. Lipid analysis (HPTLC)

At the beginning of the lipid extraction, a photo was taken of the carrier with the covered corneocytes. Using the ImageJ 1.49 v freeware (NIH, Bethesda, MD, USA; http://www.nih.gov/), the corneocyte layer was measured and identified on the sample surface. The number of cell layers on the carrier was assessed from the TEM data so that the extracted amount of lipids was referenced to a well-defined circular carrier surface (13 mm diameter) and corneocyte layers. The extraction of lipids from the corneocytes adhered on the carrier (here also referred to as slide) as well as their separation using HPTLC were carried out according to Imokawa et al. (1991) [5]. For the chromatographic analysis, Nano-Sil 20 plates (10*10 cm, Macherrey & Nagel, Düren, Germany) were used. The standard used contained the lipids cholesterol, ceramides EOS, NP and NH and free fatty acids.

The HPTLC plates were finally densitometrically measured and quantitatively analyzed.

2.5.6. Statistical analyses

All Lipbarvis® measurements were presented as mean and standard deviation, minimum and maximum, as well as quartiles. They were tested for normal distribution using the Shapiro Wilk test. In case of significant deviations from normal distribution, non-parametric methods were used for further statistical analysis otherwise parametric procedures were used.

Accordingly, measurements in Day 1 and Day 15 as well as the differences and percentage changes were compared to each other using Wilcoxon matched pairs test in case of significant deviations from normal distribution otherwise matched samples *t*-test was carried out. All tests performed were two-sided with a significance level of 5%. As the study was exploratory in nature, no adjustments for multiple testing were made. Statistical analyses were performed with SPSS Statistics 23 software (SPSS Inc. an IBM Company, Chicago, IL).

2.6. Ethics approval for clinical trials

All clinical studies were performed in accordance with the ethical principles of the Declaration of Helsinki. Prior to study start, full approval for the protocols, and protocol amendments (if applicable), was given by the local ethics committees. Written informed consent was obtained from all patients and volunteers or their respective parents/legal guardians before entry into the study.

3. Results

3.1. In vitro study

First, we investigated the putative anti-inflammatory effects of the Ec. extract *in vitro*. To mimic inflammatory conditions, human, immortalized HaCaT keratinocytes were treated with poly-(I:C), a well-known pro-inflammatory agent [13] (20 μg/ml) for 3 h with or without the Ec. extract or vehicle. We found that the Ec. extract was able to significantly reduce mRNA expression of *IL-6* and *IL-8*, two key pro-inflammatory cytokines known to be involved in a wide variety of cutaneous inflammatory processes [29,30] (Fig. 2A). Moreover, we could also demonstrate that not only mRNA levels, but also the biologically even more relevant release of these mediators was significantly suppressed by the co-administration (Fig. 2B–C), suggesting that the investigated Ec. extract may possess substantial anti-inflammatory activity *in vivo* as well, especially, since the effects were also evident following longer-term (i.e. 24-h) experiments (Fig. 2D).

Encouraged by the promising *in vitro* data, next we tested the effects of the Ec. extract containing formulation in different clinical studies in order to i) assess the safety of its application in comparison with the already successfully marketed Linola® body lotion (**Clinical Study 1**); ii) investigate its clinical efficiency in AE in comparison with a commercially available, potent comparator product (Imlan® Creme Pur; **Clinical Study 2**); and iii) explore how Ec. extract influence the structure of the epidermal lipid barrier (**Clinical Study 3**).

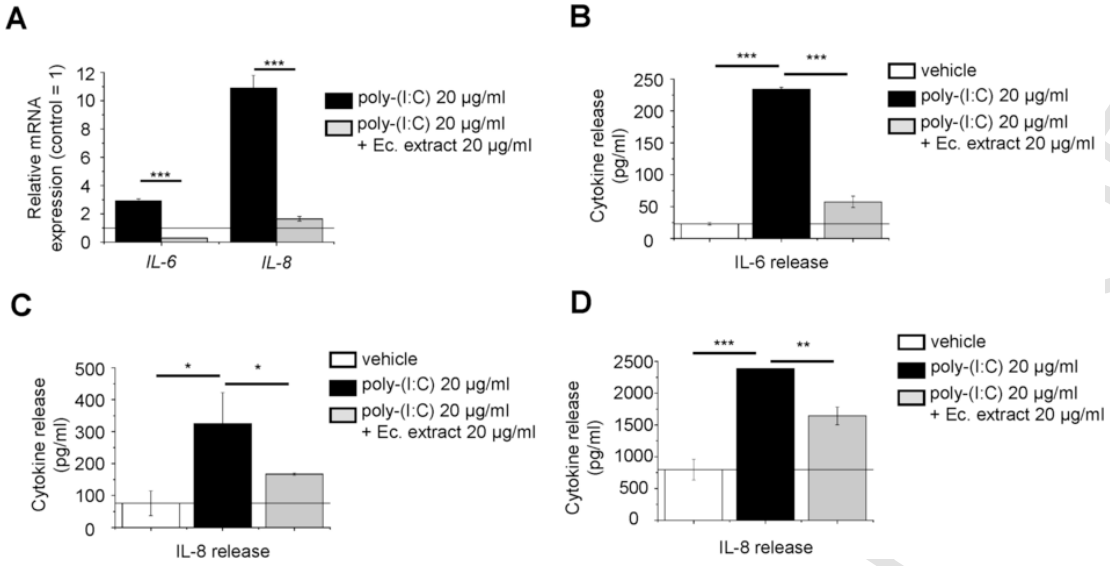

Fig. 2. *Echinacea* extract efficiently suppresses poly-(I:C)-induced pro-inflammatory cytokine expression and release from HaCaT keratinocytes. (**A**) Q-PCR analyses of HaCaT keratinocytes following the indicated 3-h simultaneous treatments. Data are presented by using the ΔΔCT method regarding 18S RNA-normalized mRNA expressions of the vehicle control as 1 (solid line). Data are expressed as mean ± SD of 2–3 determinations. One additional experiment yielded similar results. (**B**–**D**) Determination of the released cytokine concentration following 3- (**B**–**C**) or 24-h (**D**) simultaneous treatments. Data are presented as mean ± SD of 2–3 determinations. One additional experiment yielded similar results. *, **, and *** mark significant ($P < 0.05$, 0.01, and 0.001, respectively) differences as indicated.

3.2. Clinical study 1: irritancy and sensitization potential

3.2.1. Irritancy potential analysis

Evaluation of the HRIP test revealed that both Linola® body lotion and Ec. extract WO 3260 showed a low to moderate irritancy potential. Their respective Mean Cumulative Irritation Scores were 1.53 and 1.78, and there were no significant differences between test products. Moreover, there was no difference between the test products in other signs of irritation (i.e. pruritus and papules) either.

3.2.2. Sensitization potential analysis

Following a 48-h continuous occlusive application, none of the products induced erythema grade of 1 or higher on Days 24 and 26 indicating lack of clinically relevant sensitization. Consequently, the products were considered to have a low sensitization potential. Therefore we continued our experiments by evaluating clinical efficiency of WO 3260 compared to a potent, commercially available comparator product Imlan® Creme Pur.

3.3. Clinical study 2: clinical efficacy in patients with AE

3.3.1. Per-protocol population

Compared with baseline local SCORAD, both WO 3260 and the comparator resulted in a significant improvement at Day 29 ($p < 0.001$ or $p < 0.001$, respectively) and Day 57 ($p < 0.001$ and $p = 0.008$, respectively). However, at Day 85, only WO 3260 showed a significant improvement in local SCORAD versus baseline ($p = 0.013$). Moreover, this improvement was significantly different as compared to the reference product ($p = 0.047$). Results for local SCORAD are shown in Table 2 and Fig. 3. In addition, the mean local SCORAD area under the curve (AUC) was significantly lower for WO 3260 compared to the reference product (654.0 vs. 708.5; $p = 0.021$). Local SCORAD AUC outcomes are presented in Table 3.

Application of both products resulted in significant improvements in the pruritus subscore at Day 29, Day 57 and Day 85, compared with baseline ($p < 0.001$ for both products at all time points). However, WO 3260 was associated with a significantly greater improvement from baseline in the pruritus subscore already at Day 57, com-

Table 2
Local SCORAD outcomes in the per-protocol population (Clinical trial 2).

Parameter	Time					p-values of paired *t*-test		
		Mean values		Mean changes from baseline		Changes from baseline		Changes from baseline, A vs. B
		A	B	A	B	A	B	
SCORAD	Day 1	9.6 (N = 48)	9.8 (N = 47)					
	Day 29	7.2 (N = 46)	7.5 (N = 46)	−2.4 (N = 46)	−2.3 (N = 46)	<0.001*	<0.001*	0.489 n.s.
	Day 57	7.1 (N = 48)	8.2 (N = 47)	−2.5 (N = 48)	−1.6 (N = 47)	<0.001*	0.008*	0.100 n.s.
	Day 85	7.9 (N = 47)	9.3 (N = 47)	−1.6 (N = 47)	−0.5 (N = 47)	0.013*	0.511 n.s.	0.047*

A: Ec. extract containing cream (WO 3260); **B:** comparator product (Imlan® Creme Pur).

* Significant difference vs. baseline, $p \leq 0.05$. n.s = not significant; SCORAD = SCORing Atopic Dermatitis.

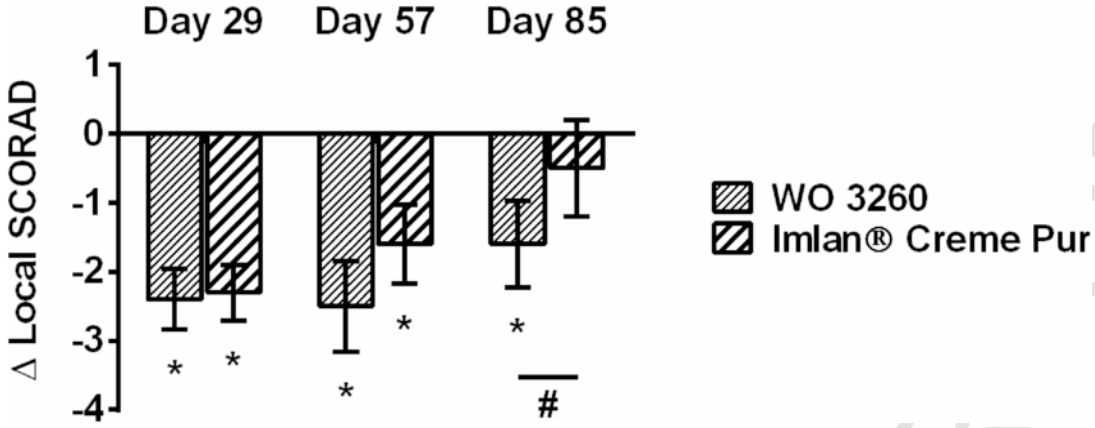

Fig. 3. Mean change (95% CI) from baseline in local SCORAD in the PP population (N = 46-48; **Clinical trial 2**). Mean change (95% CI) ±SEM from baseline in local SCORAD. * Marks significant differences; $p < 0.05$ in a matched pairs *t*-test in the comparison of measurement times on raw data; # Marks significant differences; $p < 0.05$ in a matched pairs *t*-test in the comparison of products with respect to changes from baseline.

Table 3
Mean AUC for SCORAD for PP and Results for the Comparison of Products by Wilcoxon Signed-Ranks Test (N = 44) (Clinical trial 2).

Parameter	Mean		Median		A≠B	A < B	A > B
	A	B	A	B			
AUC for SCORAD Comparison of Products by Wilcoxon Signed-Ranks Test p-values	654.0	708.5	637.0	721.0	43	28	15 0.021*

A: Ec. extract containing cream (WO 3260); **B:** comparator product (Imlan® Creme Pur).

* Significant difference, $p \leq 0.05$; SCORAD = SCORing Atopic Dermatitis.

pared with the reference product (−1.1 vs. −0.7; $p = 0.036$). In line with these data, the pruritus subscore AUC was also significantly lower for WO 3260 compared with the reference product (111.1 vs. 126.0; $p = 0.020$).

Furthermore, the use WO 3260 was associated with a significantly greater improvement from baseline in the erythema subscore at Days 57 and 85, compared with the reference product ($p = 0.022$ and $p = 0.028$, respectively). Whereas both product showed significant differences from baseline in the subscores, there were no significant differences between the two products at any of the time points for any of the other SCORAD subscores.

With respect to subjective dermatological assessments, WO 3260 exhibited a significantly greater improvement from baseline in the feeling of dryness at Day 85, compared with the reference product ($p = 0.028$), and also a significantly greater improvement in 'tickling feeling' at Days 57 and 85 ($p = 0.021$ and 0.023, respectively).

With respect to acceptance of the Ec. extract containing W/O-emulsion WO 3260, spreadability was found to be good or very good for the majority of subjects at Day 29 (89.8% or 67.4%, respectively), Day 57 (81.6% or 69.4%, respectively) and Day 85 (81.6% or 71.4%, respectively) as was the skin feeling after application of the W/O-emulsion (Day 29: 71.5% or 69.4%; Day 57: 71.4% or 61.2%; Day 85: 67.4% and 53.0%). The majority of subjects considered the greasing effect to be very good or good at all time points and they found product absorption to be fast or moderate for test and reference. Thus, having tested and proven the clinical efficiency, we finally intended to assess if our formulation impacts on the cutaneous lipid barrier.

3.4. Clinical study 3: skin barrier function

3.4.1. TEM assessment

In the morphometric analysis of the TEM images, WO 3260 resulted in a significant increase from day 1 in the number of intercellular lipid lamellae (ICLL) at Day 15 (74.9 nm/1000 nm^2 to 136.8 nm/1000 nm^2; +61.9 nm/1000 nm^2, $p = 0.001$; Fig. 4). Detailed explanation and schematic overview of the measurement can be found on Supplementary Fig. S1.

3.4.2. Lipid analysis assessment

Importantly, as revealed by careful lipid analysis, administration of WO 3260 led to significant increases from Day 1 in overall lipids at Day 15 (+9.3 ng/slide; $p < 0.001$) as well as cholesterol levels (+2.7 ng/slide; $p = 0.005$). Moreover, WO 3260 resulted in a significantly ($p = 0.002$) higher ceramide EOS (ω-esterified fatty acid + sphingosine sphingoid base) content in skin at Day 15, compared with baseline (Fig. 5). This was not observed for a linoleic acid containing water-in-oil emulsion which served as control (comparison at day 15; $p = 0.047$, Wilcoxon matched paired *t*-test). Non-significant increases from baseline in free fatty acids, ceramide NP (non α-hydroxylated fatty acid + phytosphingosine sphingoid base), and ceramide NH (non α-hydroxylated fatty acid + 6-hydroxy-sphingosine sphingoid base) were also observed for WO 3260 at Day 15.

4. Discussion

AE is one of the most common human skin diseases, exhibiting an ever increasing prevalence, especially in the industrial countries [29–31] Although it is usually not a directly life-threatening problem, by impairing quality of life of millions world-wide, it results in a significant psychological and financial burden to the society [29–31] Hence, there is an emerging demand from both the patients and the medical community to identify novel, universally efficient, yet safe medications, ideally exhibiting "favorable" side-effect profiles. To face this challenge, within the confines of the current highly focused, multidisciplinary, "bench to bedside" research project involving both *in vitro* experiments, as well as *in vivo* human clinical data, we intended to exploit and evaluate putative beneficial effects of an Ec. extract, which was already shown to potently activate the anti-inflammatory CB_2 receptors [19].

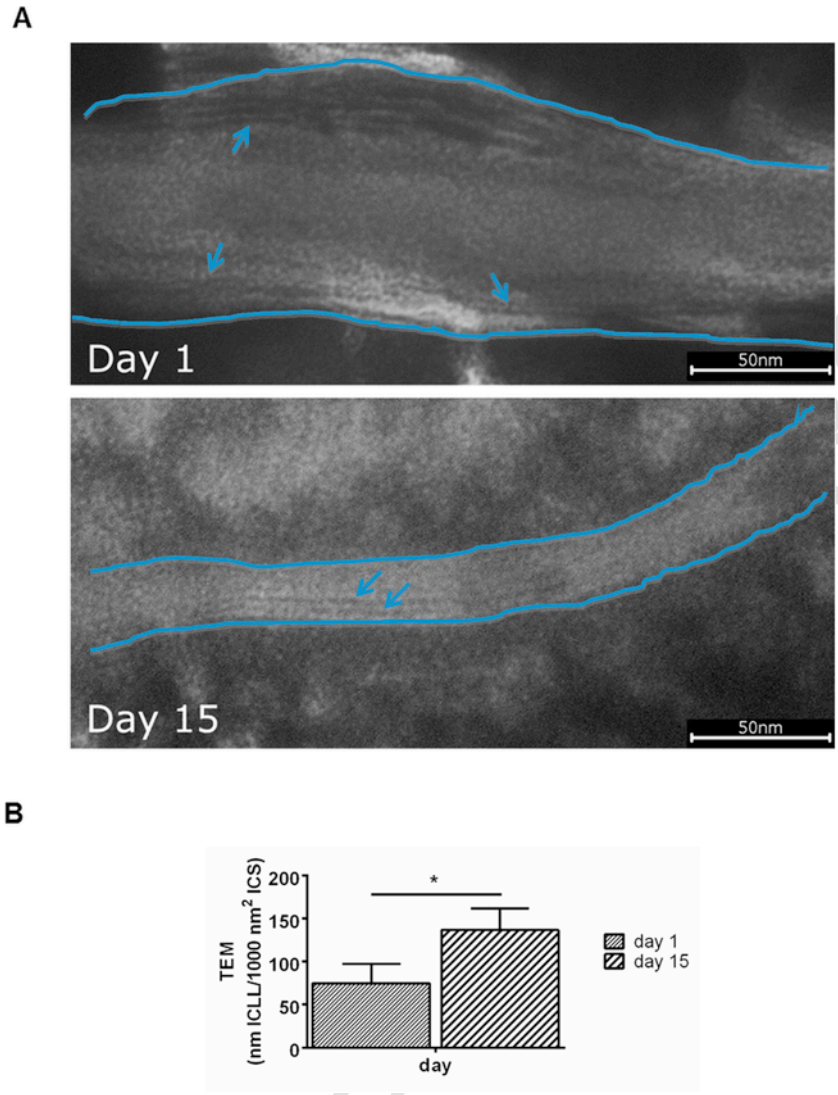

Fig. 4. Results of Lipbarvis® TEM analysis. (**A**) TEM images of the intercellular lipid lamellae (arrows) in the intercellular space (labeled between the blue lines) of stratum corneum samples at Day 1 and after treatment with WO 3260 at Day 15. (**B**) Mean values of the number of intercellular lipid lamellae (ICLL) in the intercellular space (ICS) and the results for the comparison on differences to baseline. Data are presented as mean ± SD of N = 10 patients; * marks significant differences; $p < 0.05$ in a matched pairs *t*-test.

First, in the *in vitro* study, anti-inflammatory activity of Ec. extract in human keratinocytes was demonstrated: we found significant reductions in poly-(I:C)-induced mRNA expression, as well as in release of pro-inflammatory cytokines (IL-6 and IL-8) (Fig. 2A–D). These outcomes are similar to those found in another *in vitro* study whereby the *Echinacea* spp.-derived cannabinoid receptor-binding alkylamides ("A1" and "A2") as well as the *Echinacea* spp.-derived non-CB_2 binding alkylamide undeca-2E-ene,8,10-diynoic acid isobutylamide ("A3") significantly inhibited LPS-induced TNF-α, IL-1β and IL-12p70 expressions (5–500 nM) in a CB_2-independent manner at low nanomolar concentrations; weak modulation of levels of αCD3/PMA- (phorbol-12-miristate-13-acetate; a well-known activator of classical and novel protein kinase C isoforms) and αCD28/PMA-stimulated T-cell-derived cytokines was also observed [19].

Having the encouraging data of the *in vitro* experiments in hand, an Ec. extract containing W/O emulsion was formulated and investigated regarding the safety and irritancy potential in a randomized, double-blind, single-center clinical study by HRIP test (**Clinical study 1**). Importantly, WO 3260 demonstrated an acceptable and generally low allergenic potential in a study of volunteers, similar to the already marketed, and clinically well-tolerated Linola® body lotion and placebo skin creams.

Since WO 3260 may be used two to three times daily, sun exposition of skin treated with the future cosmetic product could happen. Thus, it is also important to emphasize that none of the known ingredients of the Ec. extract containing emulsion was listed as known phototoxic or photoallergenic agent so far. Indeed, although certain extracts of *E. purpurea* (as well as others *Echinacea* spp.) were shown to possess UV-mediated activity leading to significant antifungal effects [32], these compounds were also devoid of phototoxic activity on human skin, and none of the patients reported sunlight-related side-effects [33].

Next, we assessed long-term effectiveness of WO 3260 in reducing local dermatological symptoms associated with AE (erythema, edema/papules, weeping/crusts, excoriation, lichenification, dryness, pruritus) in a 3-month prospective, randomized, double-blind, single center clinical study involving subjects with subacute or chronic AE (**Clinical study 2**). The comparator cream, Imlan® Creme Pur, also

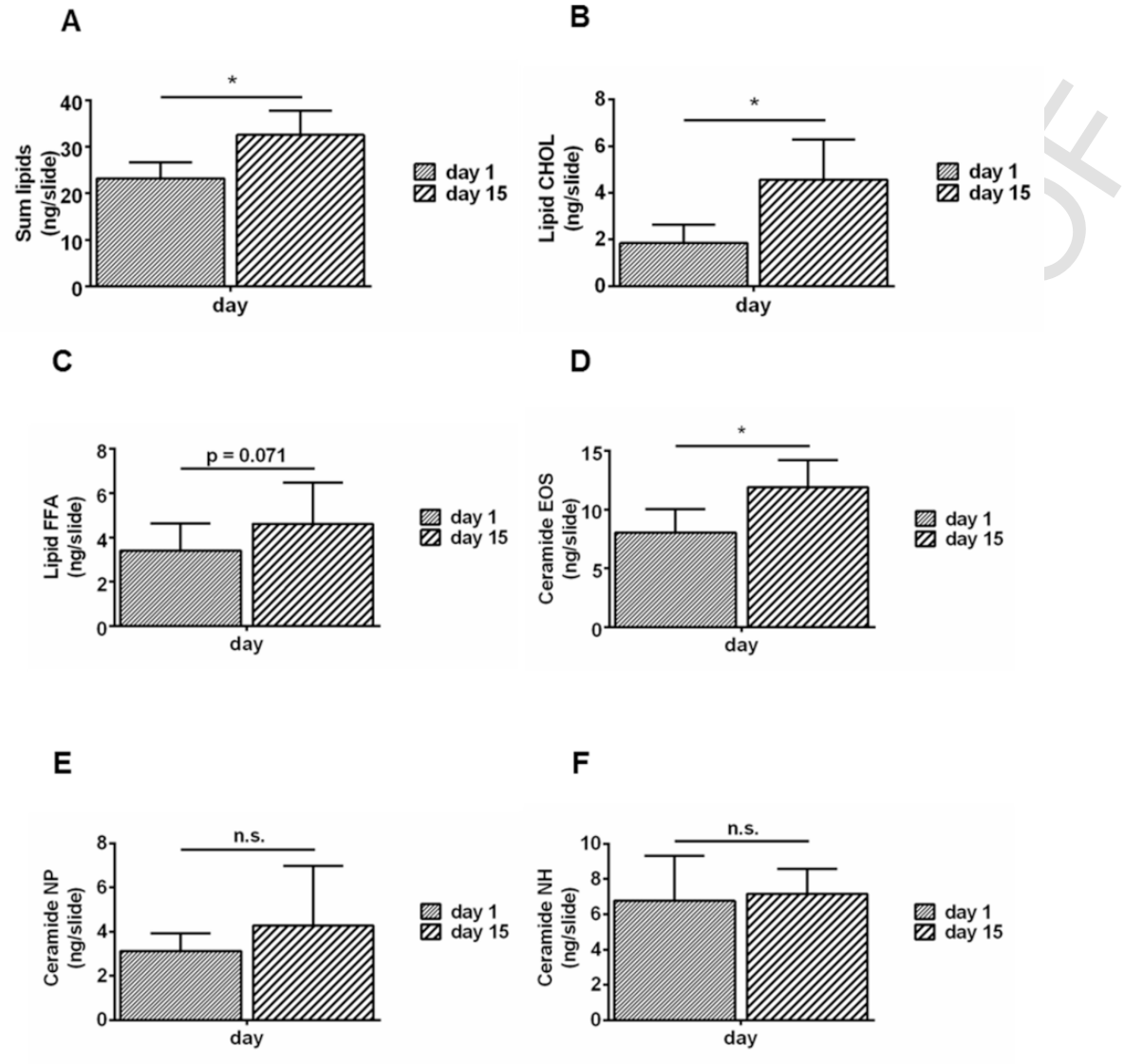

Fig. 5. Determination of lipids in skin using the Lipbarvis® LIPID analysis method. Change from baseline at day 15 in (**A**) sum of lipids, (**B**) cholesterol, (**C**) free fatty acids, (**D**) ceramide EOS, (**E**) ceramide NP, (**F**) ceramide NH. Data are presented as mean ± SD of N = 9 patients; * marks significant differences; $p < 0.05$ in a matched pairs *t*-test.

improved local symptoms, but the effects became weaker after 2 months (Tables 1–3; Fig. 3). Similar short-term improvements with Imlan® Creme Pur were reported in a study of patients with chronic dry itchy skin [25]. In contrast, improvements in AE symptoms following application of WO 3260 were sustained for up to 85 days, suggesting that the effects were due to more than mere emollient properties.

Indeed, assessing effects of WO 3260 on skin barrier, levels of overall lipids, ceramide EOS, and cholesterol were significantly increased from baseline at Day 15 following a twice-daily application as were the numbers of intercellular lipid lamellae in the intercellular spaces (Supplementary Fig. S1; Figs. 4–5). Increased ceramide EOS levels in the skin barrier have important implications for ameliorating symptoms of AE as well as for preserving skin barrier integrity. In another study assessing the effects of an *E. purpurea* extract-based cream and gel at 1 month, corneometer hydration indices increased by 10.6 AU and 11.4 AU, respectively, and wrinkles were found to decrease by 9.47% and 14.92%, respectively [34]. These results indicate that long-lasting beneficial effects of Ec. extract containing WO 3260 cream may be mediated not only by mere emollient effects, but also by (most probably CB_2-mediated) anti-inflammatory actions, as well as by improvement of the epidermal lipid barrier. However, positive regulation of cutaneous lipid production by CB_2 is not unprecedented, since this receptor has already been shown to promote sebaceous lipid synthesis of human sebocytes [35]

On the other hand, it is also important to note that other clinical data also argue for the beneficial effects of topically applied cannabinoids in AE. Indeed, an adelmidrol (a novel, palmitoylethanolamide [PEA] analogue cannabinoid receptor-targeting aliamide) containing topical emulsion, resulted in complete resolution of lesions in a small pilot study (N = 20) of patients with mild AE [36]. Furthermore, in a larger cohort study (N = 2456), a PEA containing cream resulted in complete resolution of pruritus in 38.3% of patients and significant improvement in a further 41.0% [37], whereas another study reported a significant reduction in histamine-induced itch and vasodilation following application of topical cannabinoid receptor agonists [38]. Therefore, our study adds to the growing body of evidence demon-

strating that administration of compounds targeting the endocannabinoid system may be a valid approach for patients with symptoms associated with AE and maybe other inflammatory skin diseases as well.

5. Conclusion

Here we presented *in vitro* as well as clinical data evidencing that a proprietary *E. purpurea* extract formulated in a W/O emulsion exerts clinically relevant anti-inflammatory effects, alleviates cutaneous symptoms and improves epidermal lipid barrier of AE patients. Thus, this *E. purpurea* extract-containing W/O-emulsion is a very promising product for daily medical skin care in subjects with AE.

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Conflicts of interest

This study was supported by an industrial research grant (see Acknowledgements), and three of the authors (MS, UK and CA) are employees of the sponsor. MS, UK and CA are named as inventors on a patent application for a preparation containing extracts from *Echinacea* and linoleic acid derivatives.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2017.05.015.

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