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Effect of *Physalis angulata* leaf extract cream on Interleukin-4, Interleukin-6, and Immunoglobulin-E in mice with induced atopic dermatitis

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ABSTRACT

BACKGROUND

The prevalence of atopic dermatitis (AD) and allergic or irritant contact dermatitis has been increasing significantly in the general population. Interleukin- 4 (IL-4), interleukin-6 (IL-6), and i*mmunoglobulin E* (IgE) play a key role in the pathogenesis of AD. *Physalis angulata* (PA) leaves reportedly have anti-inflammatory effects by impeding IL-4, IL-6, and IgE. This study aimed to evaluate the effect of PA leaf extract cream on IL-4, IL-6, and IgE using 2,4-dinitrochlorobenzene (DNCB) to induce AD-like skin inflammation in a mice model.

METHODS

This study used an experimental design involving 30 BALB/c mice, that were randomized into 3 groups: 1) control group receiving no treatment; 2) Vehicle treatment group receiving vehicle cream preparation; 3) PA treatment group receiving 10% PA leaf extract cream after induction of AD-like skin inflammation by DNCB. After 30 days, tissue samples were extracted from the skin lesions to measure IL-4 and IL-6 levels, and serum to measure IgE using ELISA. One-way Anova, Kruskal-Wallis and Mann-Whitney tests were used to analyze the data.

RESULTS

Group 3 (PA treatment) had significantly lower IL-4 (281.15 ± 43.14 pg/mL) than group 2 (vehicle cream treatment) (388.89±135.88 pg/ml) (p=0.001). However, although IL-6 and IgE levels were lower in group 3 than in group 2, the differences were statistically not significant (p=0.096 and p=0.479 respectively).

CONCLUSION

There were lower levels of IL-4, IL-6, and IgE in the group receiving PA leaf extract cream than in the group receiving vehicle cream preparation. Therefore, PA leaf extract cream may have therapeutic potential in AD.

Keywords: Atopic dermatitis, *Physalis angulata*, BALB/c mice, dinitrochlorobenzene

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with characteristic symptoms and clinical signs, such as itching, erythema, oozing/crusting, excoriations, lichenification, and dry skin. This condition is caused by inflammation of the skin as a result of increased histamine and inflammatory cytokine levels.[1-3] According to the World Health Organization Global Burden of Diseases at least 230 million people worldwide suffer from AD.[4] The prevalence of AD in children is about 10% to 20% whereas in adults it is about 5-10%.[5]

Atopic dermatitis is marked by infiltration of the inflammatory cytokine interleukin-4 (IL-4) producing cells in the skin and high levels of IL-4 in peripheral blood. The high IL-4 levels stimulate Th2 cells and provide positive feedback causing these cells to synthesize more IL-4.[6] Enhanced regulation of Th2 immunity can also cause maturation of B cells and differentiation of plasma cells, resulting in IgE hypersecretion and release of mast cells that will aggravate AD.[7] The presence of damaged keratinocytes disturbing the skin barrier, as well as an increased number of macrophages, cause increased secretion of IL-6, which then triggers Th2 differentiation and inhibits Th1 polarization.[8]

Topical immunosuppressant steroids or calcineurin inhibitors are the most frequent medications used because of their rapid effect in repairing AD lesions. However, the prolonged use of steroids can cause various side effects such as skin atrophy, telangiectasia, acne, and hypertrichosis. Therefore, the development of new pharmacological agents as a safe alternative therapy is very much needed, considering that AD is chronic and recurrent.[9,10] Currently, there have been many developments in various types of therapy that can target the specific mediators involved in the pathogenesis of AD. These targets include IL-4, IL-13, IgE, IL-6, B cells, IL-5, IL-31, Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT), phosphodiesterase -4 (PDE-4), IL-12, IL-17, IL-23, IL-22, histamine H4 receptor (H4R), thymic stromal lymphopoietin (TSLP) and peroxisome proliferator-activated receptor-gamma (PPAR-γ).[11]

Physalis angulata (PA) reportedly contains flavonoids such as quercetin and steroids such as physalin and angulatin-A.[12] Quercetin has been developed for AD treatment resulting in lower levels of nuclear factor kappa B (NF-κB), TSLP, IL-4, IL-6, and IgE.[10] The steroid content of PA such as physalin has also been much studied as an anti-inflammatories agent that can inhibit various types of proinflammatory cytokines such as NF-κB and IL-6.[13] *Physalis angulata* was reported to have a mechanism of action on the skin as an immunomodulator and anti-inflammatory agent with effects similar to corticosteroids and was suggested to be able to increase wound healing factors but not to reduce tumor growth factor β1 (TGF-β1) production in normal human keratinocytes, in contrast to corticosteroids which can inhibit the wound healing process and cause skin atrophy. Topically applied *Physalis angulata* is also reported as a new and innovative phytopharmaceutical with various pharmacological effects that are potentially useful to protect human skin, especially against inflammation.[14] Another study also showed that PA cream containing physalin-E conferred benefits as an anti-inflammatory agent in acute and chronic dermatitis in experimental animals, which has therapeutic potential for the chronic and recurrent inflammatory disease AD.[15] Our study might give suggestions about whether *Physalis angulata* is a potential anti-inflammatory drug. The objective of this study was to evaluate the effect of PA leaf extract cream against levels of IL-4, IL-6, and IgE in the development of a BALB/c mouse model that resembles AD with 2,4-dinitrochlorobenzene (DNCB) induction.

METHODS

Study design

This was an experimental study using an animal model with a post-test only control group

design. This study was conducted at the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Universitas Brawijaya, Dr Saiful Anwar General Hospital, Malang from October until November 2021.

Preparation of PA leaf extract and phytochemical tests

Physalis angulata leaves were obtained from Malang, East Java. The leaves were oven-dried at 40^0C and made into a powder. Subsequently, the leaves were extracted by macerating in 70% ethanol. The maceration process consisted of soaking 200 grams of powdered PA leaves in 1 liter of 70% ethanol. After five days the solution was filtered to separate the residue from the filtrate, from which the ethanol was evaporated using a rotary evaporator, leaving only the pure extract without the ethanol.

To analyze the plant extract, phytochemical tests were carried out at the Malang Medicinal Plant Station, consisting of quantitative analysis of quercetin and qualitative analysis of flavonoids and steroids.

Preparation of PA leaf extract cream

The formulation of the cream was identical to that used in the study of Herdiana et al., [16], except for the green tea used as perfume, while the 10% w/w concentration of the PA leaf extract was identical to the study of Abdul-Nasir-Deen et al.[17] The vehicle cream had the same composition except for the PA leaf extract. The materials used in the cream were glycerin 10%, triethanolamine 2%, corn oil 20%, stearic acid 7%, cetyl alcohol 2%, methylparaben 0.1%, and distilled water 100%. The cream was prepared using Tano's method, commencing by warming up the stearic acid, corn oil, and cetyl alcohol to a temperature of 70°C. The temperature was then lowered to 65°C, and triethanolamine was slowly added (Mixture 1). Glycerin and water in separate containers were heated to 80°C, stirred, and cooled down to 35°C (Mixture 2). Mixtures 1 and 2 were combined, while being stirred manually until they increased in volume and formed a smooth cream emulsion (Mixture 3). Methylparaben and PA leaf extract were added while stirring until a smooth cream was formed.[16]

Experimental animals

The research animals were 6-week-old male BALB/c mice weighing 20-30 grams, obtained from the Bioscience Laboratory, Universitas Brawijaya (Malang, Indonesia). All mice were acclimatized for one week at controlled temperature (23±3°C) and humidity (55±15%) in a cycle of 12 hours light and 12 hours darkness. Body weight and food intake were measured once a week. The sample size was determined by the Federer formula: (t-1) (r-1) >15 (t=number of treatments; r=number of replications); (3-1)(r-1) >15; 2(r-1)>15; r>8.5. In anticipation of a drop-out rate of 10%, a total sample of 30 was obtained for the three groups.

Procedure of AD induction in mice development and treatment model

This study used 30 mice that were randomly assigned to three groups: group 1 was the control group consisting of mice that were not given any treatment; group 2 was the vehicle cream group consisting of mice with DNCB-induced AD that were given the vehicle cream preparation; and group 3 was the AD treatment group consisting of mice with DNCB-induced AD that were given 10% PA leaf extract cream. The AD in the BALB/c mice was induced with DNCB using the procedure of Lim et al.,[3] as follows: A 2x2 cm area of mouse dorsal skin was shaved on day 0 and cleaned with sterile gauze and 0.9% NaCl. Sensitization with DNCB 0.5% was done for 3 consecutive days, namely on days 1, 2, and 3 using 0.15 mL of 0.5% DNCB on the shaved dorsal skin area. Subsequently the sensitized skin was challenged on days 14, 17, 20, 23, 26, and 29 (every 3 days) with 0.15 mL of 1% DNCB in groups 2 (vehicle cream) and 3 (PA leaf extract cream) at 8:00 a.m.[3] In groups 2 and 3 on day 4 of sensitization, skin lesions resembling AD were

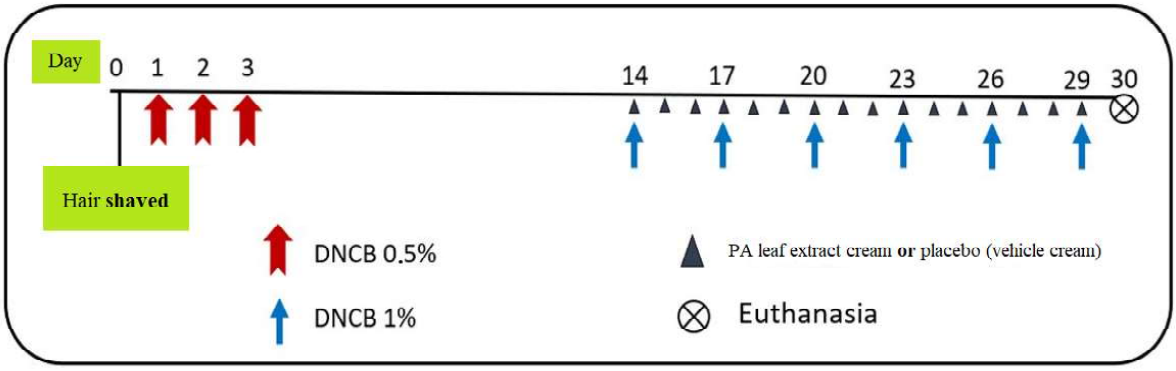

Figure 1. Induction of atopic dermatitis-like inflammation in BALB mice with 2,4-dinitrochlorobenzene (DNCB)

found, such as erythema, erosion, excoriation, scaling, and dry skin.[18]

On days 14 to 29, group 2 BALB/c mice were given vehicle cream topically on the dorsal skin every day, while group 3 was given 10% PA leaf extract cream. The respective creams were given in amounts of 0.1 g for the 2x2 cm areas on the dorsal skin of the mice with DNCB-induced AD.[3,17,19] The vehicle and PA creams were applied every day at 2:00 p.m. on the respective animals. On day 30th, the mice were euthanized by cervical dislocation. The mechanism of AD induction in the mice is depicted in the Figure 1.

Analysis of mouse serum and skin

After being left to stand at room temperature for 2 hours, each of the blood samples was centrifuged at 3000 g for 15 minutes and the supernatant was collected for determining IgE levels (ELISA kit Thermo Scientific, USA). Examination of levels of IL-4 (ELISA kit Cusabio, USA) and IL-6 (ELISA kit, Mybiosource, USA) was performed on 200 mg of skin tissue that was put in a 1.5ml Eppendorf tube, homogenized in 1 ml PBS using mortar and pestle, and stored overnight at -20 °C. The levels of each cytokine were then measured using the ELISA kits.

Figure 2. Atopic dermatitis-like lesion in 2,4-dinitrochlorobenzene (DNCB) treated BALB/c mice

Statistical analysis

Data obtained from the study was quantitative data, comprising mouse skin IL-4 and IL-6 levels, and mouse serum IgE in all three groups. Quantitative data was then processed with the Shapiro-Wilk normality test using SPSS software (v21.0). If the data was normally distributed, it was next analyzed with one-way ANOVA to find differences between the three groups. If the data distribution was not normal, the Kruskal-Wallis test was used for analysis. A p-value <0.05 was considered statistically significant.

Ethical clearance

Ethical approval of the study on BALB/c mice was obtained from the Ethics Research Commission, Brawijaya University (Animal Care and Use Committee) with the Ethical Clearance letter No. 037-KEP-UB-2021.

RESULTS

Phytochemical Results

Phytochemical testing of 70% ethanolic PA leaf extract found the presence of flavonoids, but did not find steroid compounds. On quantitative analysis, a mean quercetin level of 7.61% was obtained.

Effect of PA leaves on AD-like skin lesions induced by DNCB in mice

A total of 30 skin lesions on day four had met the criteria for lesions resembling atopic dermatitis, namely erythema, erosion, excoriation, or crusting, while repeated DNCB application induced skin lesions similar to marked AD with clearly visible erythema, excoriation, and lichenification on the dorsal skin. Healing of the observable skin lesions resulted in better looking skin, especially in the PA leaf extract cream group (group 3) compared to the vehicle cream group (group 2). Administration of PA leaf extract cream resulted in visible repair of proven lesions, accompanied by decreases in erythema, edema, lichenification, dry skin, and excoriations, although of insufficient potential compared to the mice that did not receive any treatment (group 1).

Effect of PA cream on IL-4, IL-6, and IgE levels

Based on the results of ANOVA analysis, it was found that there was a significant difference in the mean IL-4 level in the three treatment groups (p= 0.001). The mean difference between IL-4 levels in group 1 and group 2 was statistically significant (p<0.001). However, the mean difference between IL-4 levels in group 1 and group 3 was not significant (p=0.226), whereas the mean IL-4 level in group 2 was significantly different from the mean in group 3 (p=0.024) (Table 1). It was found that the p value for IgE was 0.479 (p>0.05), signifying that there was a nonsignificant difference between the groups at an error rate of 5% (Table 1). In contrast, mean IgE in group 2 was higher than in groups 1 and 3, and mean IgE levels in group 3 that was given PA cream was lower than in group 2 that was given vehicle cream preparation.

The IL-6 normality test showed that the Kolmogorov-Smirnov test value in group 3 had a p=0.007 (p<0.05), which means that it had an abnormal distribution. Then, Kruskal-Wallis and Mann-Whitney tests were performed. Analysis

Table 1. The effect of PA leaves on IL-4, IL-6 and IgE by treatment groups

	Treatment groups			p value
	Control (n=10)	DNCB + vehicle cream (n=10)	DNCB + PA cream (n=10)	
Interleukin-4 (pg/mL)[@]	216.09 ± 42,64	388.89 ± 135.97	281.15 ± 43.14	0.000
Interleukin-6 (pg/mL)[$]	4.05 (3.93-4.10)	4.17 (4.09-4.27)	4.13 (4.05-4.14)	0.011
Immunoglobulin E (ng/mL)[@]	125.04 ± 4.76	128.51 ± 7.53	126 ± 6.75	0.479

Note: Data presented as mean ± SD, except for Interleukin-6 [median (Q1-Q3)]; [@] One-way Anova test; [$] Kruskal-Wallis test; PA: *Physalis angulata*; DNCB: 2,4-dinitrochlorobenzene; Significant at p<0.05

results of the Kruskal-Wallis test obtained a p-value of 0.011 (p>0.05), signifying that although IL-6 was slightly lower in group 3 than in group 2, there was no significant between-group difference in the effect. After post-hoc analysis, only the mean difference in IL-6 between group 1 and group 2 was statistically significant (p = 0.008 <0.05). On the other hand, the mean IL-6 levels in groups 1 and 3 were not significantly different (p=0.053), while the mean IL-6 level in group 2 also did not differ significantly from that in group 3 (p=0.096) (Table 2).

DISCUSSION

The preliminary phytochemical tests of the ethanolic PA leaf extract revealed the presence of flavonoids, but not of steroids were. In this study flavonoids such as quercetin accounted for 7.16% while the steroid content was not detectable. The mice in the PA leaf extract cream group had significantly lower IL-4 levels than the mice in the vehicle cream group. However, although the IL-6 and IgE levels in the PA leaf extract cream were lower than in the vehicle cream group, the between-group difference was statistically not significant for both parameters.

Table 2. Results of Tukey multiple comparison test

Parameter	Compared Groups		p-value
IL-4[a]	1	2	0.000*
		3	0.226
	2	3	0.024*
IL-6[b]	1	2	0.008*
		3	0.053
	2	3	0.096
IgE[c]	1	2	0.462
		3	0.925
	2	3	0.691

Notes: Group 1 = no treatment, Group 2 = DNCB induced mice treated with vehicle cream, Group 3 = DNCB induced mice treated with PA cream. * Significant at p<0.05; [a]One-Way ANOVA, followed by Tukey test showed significant differences between group 1 and 2 (p=0,000) and group 2 vs. 3 (p=0.024); [b]Kruskal-Wallis test, followed by Mann-Whitney test showed a significant difference between group 1 and 2 (p=0.008); [c]One-Way ANOVA, followed by Tukey test showed no significant between-group difference

Our study results are similar to those of Abdul-Nasir-Deen et al.,[17] where the methanolic PA leaf extract, which also contained flavonoids but no steroids, reportedly still had anti-inflammatory properties and reduced paw edema in a mouse model of dermatitis. Hou et al.[20] concluded that a cream made of quercetin, which is a flavonoid, is useful in reducing AD symptoms. Quercetin cream was applied on the dorsal side of the left ears of the AD mice (C57BL/6 mice treated with topical MC903, a low-calcemic vitamin D3 analog) and reportedly reduced the expression of CCL17, CCL22, IL-4, IL-6, IFN-γ, and TNF-α.

Possible cause why our PA leaves do not contain steroids is because in our study as well as in previous studies, the fresh PA leaves came from different locations, e.g. from Bogor in the study by Herdiana et al.[16] and from Kalimantan in the study by Iswahyudi et al.[21] The place of origin of fresh PA leaves can influence the contained metabolites. The diversity of secondary metabolites is determined by various factors, such as genetics, age, season at harvest time, environmental fluctuations, soil conditions, and biochemical interactions between competing plants, that may regulate the synthesis of various secondary metabolites.[22,23] In addition, the solvents used for extracting the processed materials and the drying method (sun-, air-, or oven-drying) can also cause quantitative changes in the composition of the phytochemical compounds.[21] The quantity of the metabolites is also affected by the location or origin of the PA. Akomolafe et al.[24] from Nigeria reported that the most abundant type of flavonoid found in PA leaf extract is quercetin at 56.74 mg/g. In contrast, Nguyen and Kim [12] in Vietnam reported that quercetin is found in PA leaves at much lower concentration, in the range of 40.12-66.10 mg/kg.

Atopic dermatitis is characterized by three main features, namely abnormalities of the Th2 immune response, disruption of the skin barrier, and chronic pruritus.[25] Th2 cells produce IL-4 and IL-13, which can increase vascular adhesion,

which is molecularly related to the adhesion of eosinophils in dermatitis lesions. Th2 cells induce proliferation of B cells, which can produce IgE, which then binds to the surface of mast cells, and subsequently, mast cells release inflammatory mediators and cytokines, such as histamine, IL-6, IL-1β, or TNF-α.[3]

Lesions in AD skin are marked by an excessive expression of IL-4, IL-13, and TSLP secreted by keratinocytes.[26] The IL-4 cytokine interferes with the skin barrier and inhibits the FLG (profilaggrin) gene expression, as well as the synthesis of other proinflammatory cytokines that cause inflammation in AD. Interleukin-4 and/or IL-13 are strongly associated with three of the abovementioned AD characteristics which are key elements in the Th2 response. These cytokines could trigger and control the immune response in AD so that specific antagonism against these cytokines has developed as a therapeutic target in AD.[25,27] Interleukin-6 that is especially produced by activated macrophages, and IL-4 that is mainly released by T cells, are key mediators in the initiation and development of AD. Interleukin-6 also can influence the enhancement of T cells and B cells. The enhanced release of proinflammatory cytokines could be detected at different AD phases which shows that cytokines are closely involved in the development of AD.[28] Immunoglobulin E plays a role in AD via binding to mast cells and basophils that have high affinity IgE receptors (FCeRI), whereupon these cells will then release a signal for degranulation and cause inflammation.[29]

Atopic dermatitis affects the human immune system and causes clinical symptoms such as erythema, skin edema, excoriation, lichenification, and abnormal epidermal thickening that are observed in mice with DNCB-induced AD.[28] The immune response in mice with DNCB-induced AD can be observed from the increased serum levels of IgE, IL-4, and IL-6 in the study, and this condition is correlated with enhanced infiltration of inflammatory cells and mast cells in the dorsal skin. However, AD symptoms appear to be reduced after treatment with PA leaf extract cream for two weeks. Our study shows that PA leaf extract cream can significantly lower IL-4 levels in the skin of the DNCB-sensitized mice. The PA leaf extract cream can also lower IL-6 levels in the mouse skin and IgE levels in mouse serum compared with mice that were only given the vehicle cream preparation.

The mechanism of action of the PA leaf extract cream in AD is due to the existence of the flavonoid quercetin, as well as steroids such as physalin that have been reported by many to be able to suppress TSLP or NF-kB which is a path in the formation of IL-4 or to directly inhibit IL-4, IL-6, and IgE.[10] Abnormalities of the epidermal barrier in AD increases the enzyme kallikrein-5, which then binds to PAR2 and triggers NF-κB, which in turn can increase the release of TSLP cytokines by keratinocytes.[30,31] Thymic stromal lymphopoietin induces dendritic cells and activated Langerhans cells to migrate to the lymph nodes as antigen-presenting cells (APC) and convert naive T cells into Th2 cells.[32,33] The Th2 cells could return to the dermis and release IL-4 and IL-13 which can trigger the signs and symptoms of AD. In addition, Th2 causes a change in the IgE class produced by the B cells.[31] The immunoglobulin E that is produced then stimulates mast cells, which degranulate when bound to specific allergens and release various types of molecules including IL-4. Increasing IL-4 will also aggravate AD by reducing epidermal differentiation and downregulating AMP expression thereby increasing the risk of infection.[34]

Quercetin is a pleiotropic molecule that can be used against various molecular targets to control proinflammatory cytokines and therefore has potential for AD therapy.[35] Several studies have demonstrated that quercetin constitutes a potent anti-AD agent that can be given orally or topically. Karuppagounder et al. [36] used quercetin orally (at 50 mg/kg) for two weeks in the NC/Nga AD mouse model and reported that quercetin could lower serum IL-4 levels and modulate HMGB1/RAGE/ NF-κB signaling and Nrf2

protein induction. The study of Hou et al.[20] concludes that administration of 1% quercetin cream for seven days can reduce the expression of IL-4, CCL17, CCL22, IL-6, IFN-γ, and TNF-α in the ears of C57BL/6 mice with MC903-induced AD. The quercetin derivative quercetin-3-O-(2"-gallate)-α-l-rhamnopyranoside (QGR) has also been reported to lower the expression of IL-4, IL-5, and IL-13 when 1% QGR was given topically to NC/Nga mice for four weeks.[37] Treatment with quercetin derivatives could push the expression of proinflammatory cytokines through modulation of NF-κB, IL-4, IL-5, and IL-13, serum IgE, and levels of eosinophils, iNOS, and COX2, HMGB1, RAGE which play an essential role as inflammatory mediators. Quercetin has also been reported to inhibit the HMGB1, NF-κB, JAK/STAT, and TSLP pathways in the NC/Nga AD mouse model. In addition, it has been shown that quercetin inhibits cytokines and other pro-inflammatory agents, such as IL-1β, IL-6, and TNF, and increases IL-10 levels in liposaccharide-induced inflammation in mice.[10,36] According to the literature, quercetin can inhibit NF-κB, TSLP, and IL-4 pathways which are unique inflammatory pathways in AD. However, this does not preclude the possibility that the quercetin contained in the PA leaf extract cream can inhibit IL-4 through other pathways, in view of the fact that the pathophysiology of AD is very complex, because PA could inhibit a variety of signaling pathways or cytokines.

One of the limitations of this study is that the PA leaf extract contained no steroids, which are considered important for suppressing the inflammation of atopic dermatitis. Therefore it is necessary to search for PA leaves that contain both flavonoids and steroids, in the expectation that they can provide a more synergistic effect against the AD-like inflammation in the mice. Another limitation is that there was no comparison of the PA cream with other standard topical agents for AD, such as topical corticosteroids and calcineurin inhibitors. It is still unclear whether the improvement in IL-4, IL-6, and IgE levels due to PA leaf extract cream equals or surpasses that of topical steroids or calcineurin inhibitors. Even so, the IL-4, IL-6, and IgE levels in mice given PA cream at a concentration of 10% proved to be lower than in mice given only the vehicle cream. Further studies are needed on the proportion of the extract in different creams, on experiments with other preparations, such as ointments or gels, and on the use of solvents and other extraction methods to obtain a maximal yield of flavonoids and steroids, thus providing adequate treatment of AD lesions.

CONCLUSIONS

Physalis angulata leaf extract cream at 10% concentration can inhibit the inflammatory response in mice DNCB-induced AD lesions by lowering IL-4, IL-6, and IgE levels. The therapeutic use of PA leaf extract cream is exceedingly promising and has the potential to be developed into standard AD therapy.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS

DPE: basic concept, design, writing the manuscript and performing the experiment. SB: supervising, critical reviewing and final approval of the manuscript. WK: analyzing, interpreting data, and helping draft the manuscript. HB and

AR: critical reviewing and final approval of the manuscript. All authors have read and approved the final manuscript.

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