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Indonesian *Ciplukan* Extract Inhibited TGF-β1/NF-κB Pathway in Experimental Psoriasis Mouse Models

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Abstract

BACKGROUND: The global prevalence of psoriasis, a chronic inflammatory skin disease, has substantially increased in the past decade. The increase activity of transforming growth factor β1 (TGF-β1)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway which cause inflammation is the major pathological mechanism in psoriasis. The current psoriasis treatment using chemical agents is hampered by the side effects when used long term, which underlines the need for alternative, low side effect anti-psoriatic agents. The extract of *Physalis angulata* L., also known as *Ciplukan* in Indonesia, contains physalins, compounds known for their anti-inflammatory effects, but whose effect on psoriasis has not been studied.

AIM: This study aimed to investigate the effect of *Ciplukan* extract (CE) to TGF-β1/NF-κB pathway in psoriasis mouse models.

METHODS: This was experimental study with posttest-only control group design. The CE active ingredients were identified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Twenty-five female imiquimod (IMQ)-induced psoriasis-like dermatitis mice were allocated into five groups, with three groups receiving 7 days of 400, 800, and 1200 mg/kg bodyweight doses of CE, respectively, and two groups serving as control and IMQ groups. The NF-κB and TGF-β1 expressions were evaluated using Allred score based on immunohistochemistry (IHC) staining. Histopathology and clinical psoriasis manifestations were assessed using Baker's from hematoxylin-eosin (HE) staining and Psoriasis Area and Severity Index (PASI) scores. The Kruskal–Wallis followed by Mann–Whitney tests was conducted for data analysis. $p < 0.05$ was considered to be statistically significant difference.

RESULTS: Based on LC–MS/MS test, physalins B, D, and F were active ingredients from CE in ethyl acetate solution. An improvement in psoriasis inflammation was observed in 400 and 800 mg/kg bodyweight doses of CE, but only the dosage of 800 mg/kg BW significantly decreased of Allred scores from NF-κB and TGF-β1 expressions; Baker's and PASI scores compared to IMQ group ($p < 0.05$). The 1200 mg/kg bodyweight doses of CE associated with acute toxicity signs and mortality, meanwhile, dosage of 800 mg/kg BW showed the highest efficacy with lowest toxicity effect.

CONCLUSIONS: *Ciplukan* extract improved psoriasis manifestations through inhibition effect to TGF-β1/NF-κB pathway and the extract might be developed as an alternative anti-psoriasis agent.

Introduction

Psoriasis is an immune-mediated chronic inflammatory disease characterized by erythematous plaque covered by silvery adherent scales [1] with epidermal hyperplasia due to augmentation of keratinocyte turn over and interaction of immune cells in dermis [2]. This condition may lead to decreased quality of life and psychosocial problems for the affected persons [1]. Psoriasis prevalence may differed based on geographic, genetic, and environmental factors, but approximately over 100 million people globally suffered from this disease [3]. At present, psoriasis is still a challenge for physicians because of increase of health-care cost [4], unsatisfied, and low compliance from patients due to limited therapeutic options and long duration treatment [5]. These unmet outcomes from psoriasis treatment may be caused by the complex pathogenesis that not fully understood, so further investigations are needed.

In psoriasis, there is a complex communications between keratinocyte, dendritic cells, other immune cells, and pro-inflammatory cytokines through multi-cell signaling pathways. Two important cell signaling in psoriasis are the transforming growth factor beta 1 (TGF-β1) and *nuclear factor kappa B* (NF-κB). Both TGF-β1 and NF-κB are principal regulators in epidermal homeostasis, inflammatory responses, and carcinogenesis [6]. Although the role of TGF-β1 in psoriasis has been only inconclusively related to the inhibition and induction of keratinocytes proliferation [7],

other studies showed TGF-β1 liable to increase keratinocyte hyperproliferation in psoriatic mice model through Smad3-dependent mechanism [8], [9]. The TGF-β1 and NF-κB may form a pathway that initiated by TGF-β1 activation that causes NF-κB and its downstream stimulation to start inflammation. The canonical TGF-β signaling pathway, activation of TGF-β1, started with its bindings to receptor subunit transforming growth factor beta II (TβRII) that sequentially will activate TβRI followed by the activation of TGF-β-activated kinase 1 (TAK1) directly. The stimulated TAK1 will phosphorylate the inhibitor-kappa B kinase (IKK) complex that leads to phosphorylated, ubiquitinylated, and degraded of the inhibitor-κβα (Iκβ) by proteasome. When Iκβ is degraded, its binding to NF-κB in the cytoplasm will be disconnected so that NF-κB will be free to move from the cytoplasm to enter the nucleus. The NF-κB that successfully entered the cell nucleus will bind to DNA, meanwhile IκK phosphorylation of the RELA (p65) subunit is necessary to target genes transactivation [10]. Meanwhile, the activated NF-κB may induce keratinocytes to produce pro-inflammatory cytokines, and of LL-37, which maturates dendritic cells to release IL-23 [11], [12]. IL-23 together with TGF-β1 and IL-6 induce differentiation of Th0–Th17 cells that produce inflammatory cytokines IL-17 and IL-22 [12], [13]. *IL-17* attracts neutrophils from vascular into epidermal space, while IL-22 induces anti-apoptotic Bcl expression [14], [15]. So that it can be proved that TGF-β1/NF-κB pathway has a major role in initiating psoriasis pathogenesis that related to the psoriatic skin manifestations.

Oral methotrexate (MTX) is still the most commonly used treatment worldwide because of its affordability, effectiveness, and safety profile, but long-term use of MTX may induce liver, kidney, and hematology disorders. Furthermore, the use of MTX is contraindicated for pregnant and breastfeeding women [16]. Other therapeutic agents for psoriasis are narrowband ultraviolet B [17] and certain biologic agents [18]. However, these agents are not widely available and are relatively expensive, necessitating the development of new, safer, and more affordable anti-psoriasis agents with anti-inflammatory and pro-apoptotic effects.

Ciplukan (*Physalis angulata* L.), a member of the *Solanaceae* family, is an Indonesian wild plant that has been used as a traditional medicine for years. The previous study has shown that CE has physalins as major active ingredients of this extract [19]. Other studies have been conducted to investigate the effect anti-inflammatory of physalins B, D, and F on the inhibition of NF-κB [20], [21], [22], [23]. Physalin D from traditional China plant *Physalis alkekengi* L. showed decrease of TGF-β1 through Smad2/3 phosphorylation inhibition in male C57BL/6J *wild-type* mice [24]. However, no studies have been conducted to evaluate physalins from CE as potential alternative anti-psoriatic agents. This study aimed to investigate the suitability of CE to be developed as an anti-psoriasis treatment through TGF-β1/NF-κB pathway inhibition in psoriasis-like dermatitis in mouse models.

Methods

Study design

This is an experimental study with posttest-only control group design using BALB/c (*Mus musculus*) mice as psoriasis animal models.

Animal experiment and sample selection

Twenty-five of female BALB/c mice aged 8–11 weeks and weight of 20–25 g were used in this experiment. Mice with over than 20% of ranged weight, abnormal behavior, and skin anatomy were excluded based on veterinarian recommendations. The mice distributed in five groups using simple random sampling with five animals per group as recommended by the WHO [25]. All mice were acclimatized for 7 days in animal house of Medical Faculty, Universitas Jenderal Soedirman. After acclimatization period, all mice were have their back shaved for 2 × 2 cm^2 as treatment site using electric shaver, meanwhile, the remaining hair was eliminated with hair removal cream (Veet®, Reckitt Benckiser, Cedex, France) [26].

Each mice in the control group (A) have smeared of 62.5 mg of Noroid cream (SOHO Global Health, Jakarta, Indonesia) in the treatment site for 7 consecutive days. Meanwhile, the treatment site of mice in the positive control (B) and experimental groups (C–E) was smeared with 62,5 mg of Aldara cream (5% IMQ, 3M, Pharmaceutical, UK) daily for 1 week to induce psoriasis-like skin inflammation [27]. At the 8th day, mice in Groups A and B were sacrificed using cervical dislocation method followed by back skin harvesting to have pathology tests. Whereas, Groups C–E have additional 7 consecutive days of 62.5 mg of Aldara cream and orally CE of 400, 800, and 1200 mg/kg BW. In the 15th day, all mice in experiment groups were terminated with the same procedure as Groups A–B previously. The features of skin inflammation and health condition of all mice were evaluated everyday.

Preparation and compound identification of Ciplukan extract

Fresh of all parts from *Ciplukan* was collected from Mersi, East Purwokerto, Central Java, Indonesia, in January–June 2020. The plant materials were air-dried and powdered using grinder. The specimens were then preserved in the Department of Pharmacology,

Faculty of Medicine, Universitas Jenderal Soedirman, Indonesia. In this study, we used three different polarity solvents that are 90% ethanol, n-hexane, and ethyl acetate. The procedure of solvent extraction was at room temperature as follows: The powdered was macerated in 90% ethanol for 72 h. After filtration, the residue was macerated using n-hexane for 3 days. The residue from n-hexane filtration further macerated in ethyl acetate for 3 days duration. These extraction procedures were a modified from the previous studies [28], [29]. The extract from each solvent was dried using rotary evaporator, and the residue was sent to the laboratory to have liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Waters, Milford, MA, USA) test in Indonesian Institute of Science, Serpong, Banten, Indonesia.

Measurement of NF-κβ and TGF-β1 expressions

The immunohistochemistry (IHC) method was used to identify NF-κβ and TGF-β1, expressions through the following procedure. The paraffin block of skin tissue was cut into 4 mm sections on a poly-L-lysine slide and incubated at 37°C for 24 h. The slides were then immunostained with primary antibodies against NF-κβ p65 Rabbit pAb-polyclonal antibodies from ABclonal (Massachusetts, USA), and TGF-β1 (3C11) Santa Cruz Biotechnology Inc. (Dallas, USA), and incubated at 4°C for a further 18 h. The further procedures were completed according to the standard protocol (MP-7601; IHC Guide, Vector Laboratories). The bright-field images were captured using Optilab lens and microscope. The Allred score [30] was used to quantify the TGF-β1 and NF-κβ expressions.

Measurement of Baker's score of psoriasis pathology

The harvested skin was fixed in a 10% buffered formalin solution and suspended in paraffin. Multiple 5 mm sections of paraffin block were then taken by a microtome. The deparaffinization was done using a sequential xylene wash, and serial baths in ethanol were used to rehydrate skin tissue sections.

Table 1: Baker's score to measure psoriasis pathological features [31]

Items	Score
Keratin	
Munro abscess	2.0
Hyperkeratosis	0.5
Parakeratosis	1.0
Epidermis	
Thinning above papillae	0.5
Lengthening and clubbing of rete ridges	1.5
Acanthosis	0.5
Lack of granular layer	1.0
Dermis	
Lymphocytic infiltrate	
Mild	0.5
Moderate	1.0
Marked	2.0
Papillary congestion	0.5

The HE staining was performed following standard protocol from Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Negeri Sebelas Maret, Surakarta, Indonesia. The slides were then observed under a light microscope (Olympus, Tokyo, Japan) to rated Baker's score (Table 1) by two certified pathologists [31].

Measurement of skin inflammation severity score

The inflammation on the treatment site induced by IMQ was marked by erythema, scaling, and thickness, and was evaluated according to the modified PASI score for mice [26]. The scoring process was conducted by two certified dermatologists, with the scores for each inflammation sign defined and ranged from 0 to 12 as described previous study [26].

Ethics approval

This study design, including all experimental procedures, has been reviewed and approved by the Health Research Ethic Committee, Faculty of Medicine, Universitas Jenderal Soedirman with reference number: 205/KEPK/IX/2020.

Statistical analysis

The PASI, Baker, and Allred scores were described as mean and standard deviation (SD). The difference of the scores between the control and treatment groups was tested using the Kruskal–Wallis test. The Mann–Whitney U-test was conducted *post hoc*. The significance level was set at 0.05, such that $p < 0.05$ was considered to be statistically significant difference.

Results

CE active ingredients identification

The result from LC–MS/MS test (Waters, Milford, MA, USA) showed *Ciplukan* extract in ethyl acetate expressed three kinds of physalins that are physalin B (PubChem: 5-hydroxy-2,9,26-trimethyl-3,19,23,28-tetraoxaoctacyclo[16.9.1.118,27.01,5.02,24.08,17.09,14.021,26]nonacosa-11,14-diene-4,10,22,29-tetrone), physalin D (Pubchem:5,14,15-trihydroxy-2,9,26-trimethyl-3,19,23,28-tetraoxaoctacyclo[16.9.1.118,27.01,5.02,24.08,17.09,14.021,26]nonacos-11-ene-4,10,22,29-tetrone, and physalin F (PubChem: 5beta,6beta-epoxy-5,6-dihydrophysalin B). Only *Ciplukan* extract in ethyl acetate was used further in this study because it has three physalins whereas the

other solvent extracts showed two physalins. *Ciplukan* extract in ethyl acetate was diluted in sterile water to produce 400, 800, and 1200 mg/kg BW.

Signs of acute toxicity

Signs of acute toxicity were observed in the group treated with 1200 mg/kg BW. These three mice showed inactive behavior, ruffled fur, and a decrease of body weight over than 20% followed by death beginning on the 4th day of treatment. Dissection revealed the internal organs to be swollen and discolored (data not shown). Death mice were replaced by the same condition of mice until research requirement was fulfilled.

The mean Allred, Baker's, and PASI scores between groups

This study results demonstrate CE's gave refinement effects as represented by the improvement in Allred scores for NF-κβ and TGF-β1 expressions, and the scores of Baker and PASI among the treatment groups displayed in Table 2.

Table 2: Measures of Allred, Baker's, and PASI scores from all groups

Groups	Parameters			
	Means ± SD of Allred scores for expression of		Means ± SD of Baker's score	Means ± SD of PASI score
	TGF-β1	NF-κB		
Control	0.00 ± 0.00	3.20 ± 1.30	0.70 ± 0.67	0.00 ± 0.00†
IMQ	4.00 ± 0.00	6.40 ± 0.89	5.20 ± 1.79	8.80 ± 1.64†
CE 400*	3.40 ± 1.34	5.00 ± 2.35	4.80 ± 1.58	0.80 ± 0.45††
CE 800*	1.40 ± 1.34	4.00 ± 1.00	3.30 ± 1.60	0.20 ± 0.45††
CE 1200*	4.00 ± 0.71	5.60 ± 0.55	1.30 ± 0.27	0.60 ± 0.55††

*mg/kg BW; †measured at day 7; ††measured at day 14.

NF-κβ and TGF-β1 expressions analysis using IHC staining

Table 2 shows the comparisons of NF-κβ and TGF-β1 expressions based on Allred scores among groups. Findings from this study indicated that the administration of 400 and CE 800 mg/kg BW doses corresponded to reduced NF-κβ and TGF-β1 expressions, although only CE 800 mg/kg BW dose showed the significantly decreased ($p < 0.05$) of NF-κβ and TGF-β1 Allred scores compared to IMQ group (Figure 1). Meanwhile, CE 1200 mg/kg BW appeared to have NF-κβ and TGF-β1 Allred scores that similar to IMQ group where severe psoriasis features were manifested. The IHC staining also showed low nucleocytoplasmic NF-κβ and TGF-β1 expressions in all epidermal and dermal layers except in IMQ and CE 1200 mg/kg BW groups (Figure 2).

Histopathological analysis using HE staining

From Table 1, the application of IMQ to the treatment site caused remarkable psoriatic

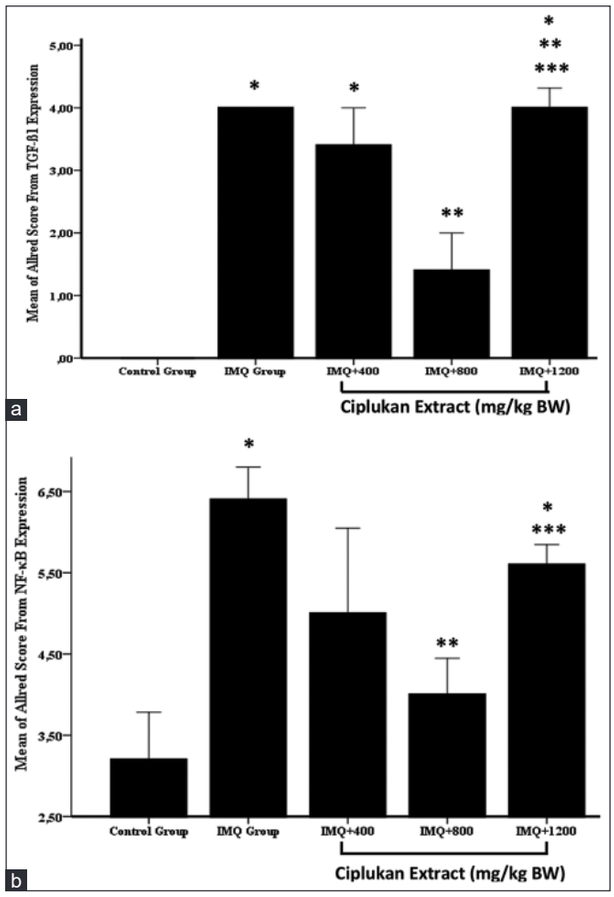

Figure 1: The Kruskal–Wallis test showed significant differences in the means ± SD of Allred scores from TGF-β1 (a) and NF-κB (b) expressions among the groups. The Mann–Whitney U-test showed significant differences of these protein expression between IMQ group compared to the control group (*). Only CE 800 mg/kg BW improved all expressions that similar to the control group and had significant differences compared to the IMQ group (**). Compared to other doses of CE, the CE 1200 mg/kg BW exhibited higher TGF-β1 and NF-κB expressions that similar to the IMQ group and significantly differed to CE 800 mg/kg BW (***). The significant differences between parameters were achieved at $p < 0.05$

histopathologic changes as indicated by the highest mean Baker's score. Classic histopathological psoriasis such as Munro microabscess, epidermal hyperplasia, acanthosis, parakeratosis, diminished suprapapillary, dilated capillaries, and lymphocyte infiltration was also clearly observed in the IMQ group (Figure 2). The administration of CE correlated with an improvement in histopathological psoriasis features, with the 1200 mg/kg BW treatment group displaying the greatest changes, comparable to the histological features of the control group (Table 1 and Figure 3).

The clinical changes of psoriasis-like dermatitis in mouse models

IMQ exposure for 7 days on the treatment site successfully induced skin lesions mimicking the peak of psoriasis-like dermatitis mice that were erythematous, thick, and scaly. On the 7th day of IMQ application, the

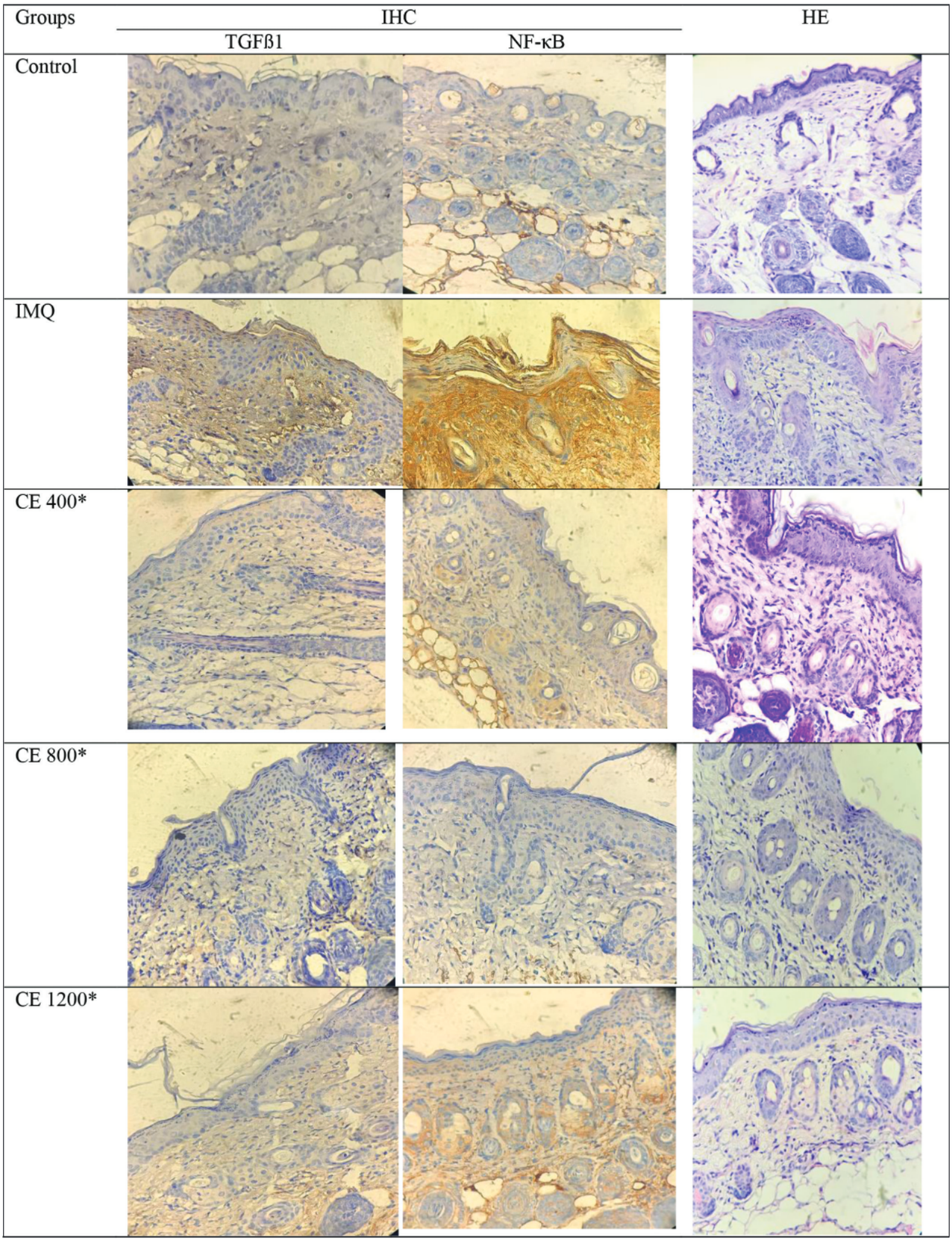

*Figure 2: The IHC and HE staining from psoriasis mouse model skin. The groups of CE dose 800 mg/kg BW showed significant improvement of TGF-β1 and NF-κB expressions; only CE dose 1200 mg/kg BW represented refinement in histopathological features similar to that of the control group although this group expressed TGF-β1 and NF-κB almost the same to the IMQ group. * = mg/kg BW*

mean PASI score was substantially increased in all treatment groups and significantly differed ($p < 0.05$) from that of the control group (Table 1 and Figure 4a). The administration of IMQ and CE in the next 7 consecutive

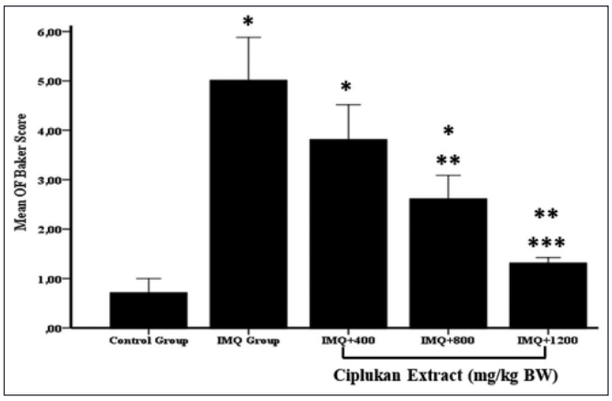

*Figure 3: The effects of CE on histopathological features assumed from Baker's score. The IMQ, CE 400, and CE 800 mg/kg BW groups showed significantly higher Baker's scores compared to the control group (Mann–Whitney U-test; *). The Baker's score of CE 1200 mg/kg BW significantly decreased compared to IMQ (**), 800 mg/kg BW groups (***) and demonstrated similarity to the control group. The significant differences were at the level of p < 0.05*

days to the treatment groups showed to reduce the PASI scores marked by diminished of erythematous, thick, and scaly skin, and the CE 800 mg/kg BW treatment group appeared to show the lowest PASI scores (Table 1 and Figure 4b). The differences in the clinical features of psoriasis between groups before termination are shown in Figure 5. The IMQ group associated with severe psoriasis manifestations; meanwhile, CE 800mg/kg BW dose revealed amelioration these manifestations clearly (Figure 5).

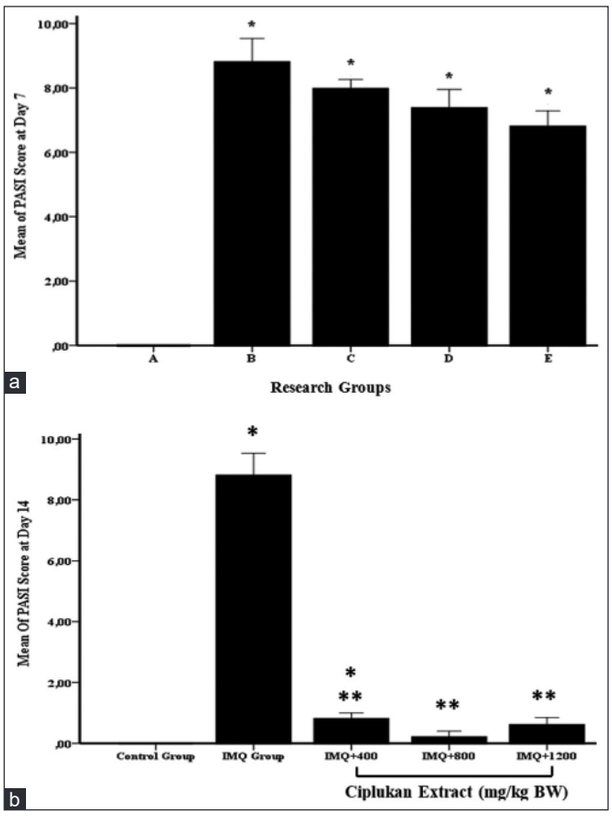

*Figure 4: Psoriasis clinical manifestations. The Kruskal–Wallis test demonstrated significant different in the means of PASI score between the groups. A 7-day IMQ applications significantly increase PASI score of treatment groups (B-E) compared to A group (a; Mann–Whitney U-test; *). All doses of CE exhibited significant decrease of PASI score compared to the IMQ group, but CE 800 mg/kg BW showed the lowest score compared to other doses (b; Mann–Whitney U-test; **). The significant differences were determined at p < 0.05*

Discussion

This study aimed to assess the efficacy of CE to be developed as an anti-psoriasis agent, indicated by TGF-β1/NF-κβ pathway inhibition in psoriasis-like dermatitis in mouse models. Findings from this study were consistent with previous findings in that the application of IMQ successfully induced both clinical and pathologic manifestations of severe psoriasis. Psoriasis features from IMQ-induced GILZ-Tg mice [32] and keratin 5 *promoter* (K5. TGF-β^{WT}) that treated with SIS3 or DMSO showed cutaneous TGF-β1 increment [9]. Earlier studies have been shown that IMQ may cause psoriasis-like features in mice through toll-like receptor 7 (TLR7) activation that leads to increase NF-κβ expression [33]. In this study, we can prove that IMQ application to mice may activate TGF-β1/NF-κβ pathway that is related to severe psoriasis manifestations. Our study results also indicated that the administration of CE showed to improve psoriasis, based on indicators such as the decreased of TGF-β1 and NF-κβ expressions that may show TGF-β1/NF-κβ pathway inhibition, as well as improvement in Baker and PASI scores. However, the administration of CE in a 1200 mg/kg BW dosage appeared to induce acute toxicity.

Previously, the role of TGF-β1 in psoriasis has been only inconclusively related to the inhibition and induction of keratinocytes proliferation. This study confirms findings from a previous study, in which TGF-β1 expression was linked to psoriasis inflammation [34]. Although evidence of the effect of physalins on TGF-β1 is still limited, an earlier study showed that physalin D from *Physalis alkekengi* L. may inhibit Smad2/3 phosphorylation, which leads to the reduction of TGF-β1 activation in male C57BL/6J wild-type mice [24]. This may explain the apparent ability of CE to reduce TGF-β1 expression in our study as seen from IHC staining and Allred scores, as CE contains physalin D as one of the active ingredients.

Findings further showed that CE is effective in improving inflammation conditions in psoriasis mouse models. This may relate to the role of physalins B, D, and F as the active ingredients of CE. A previous study showed that physalins stabilize IκBα, maintaining the binding of NF-κB to IκBα in the cytoplasm [20]. Physalin D has been shown to effectively reduce NF-κB receptor

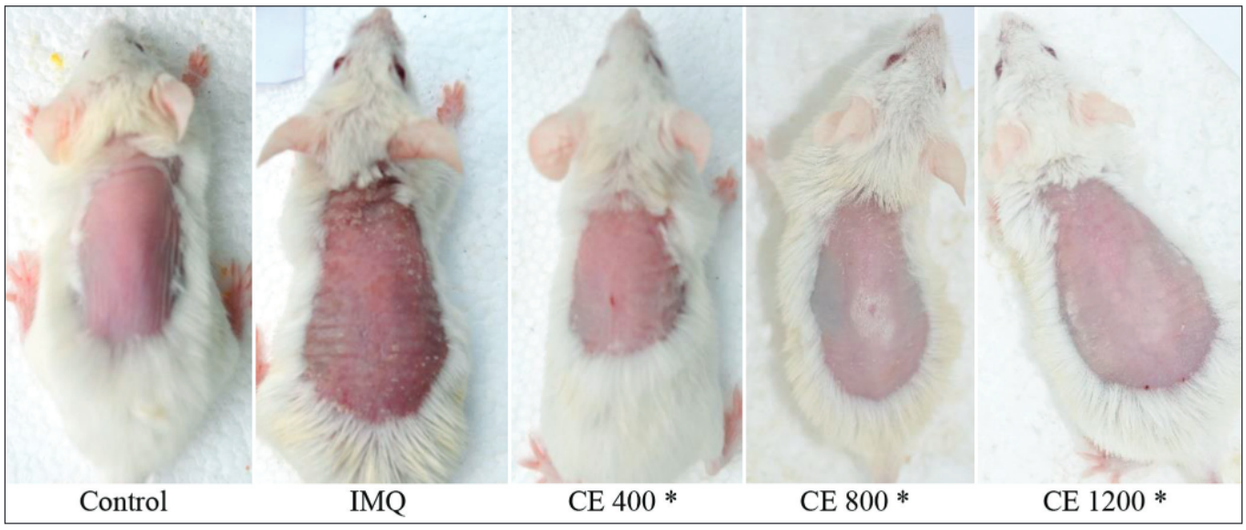

*Figure 5: Psoriatic skin lesions were improved by CE with dose of 800 mg/kg BW that gives similar improvement to the control group. *mg/kg BW*

activation, leading to the inhibition of osteoclast genesis and bone loss in rats [22]. Wu *et al.* (2012) showed physalin F reduced NF-κB activation based on dose and time period exposure in renal cancer cell [23]. Physalins have been proven to hindered IκBα degradation that followed by impeded NF-κB translocation from cytoplasm into nucleus [20]. Physalins B, D, and F as active ingredients from CE in this study have the same mechanism as the previous studies report that resulted with decreased of NF-κB expression. Furthermore, the decreased of TGF-β1 may correlate to inhibit NF-κB activation by inactivation of TAK1, IKK complex phosphorylation, and Iκβ degradation. Our study clearly showed that CE ameliorated psoriasis-like dermatitis in mouse models by inhibiting TGF-β1/NF-κB pathway.

Although other studies have reported that the effectivity of physalins is dose and time dependent, our study demonstrated that CE at a dosage of 800 mg/kg BW was the most effective in improving psoriasis parameters, compared to other doses. This study also revealed that despite significant improvement in the psoriasis manifestations of CE-treated groups, toxicity signs were observed at the highest dose (1200 mg/kg BW). These signs included inactive behavior, ruffled fur, body weight loss, and visibly abnormal internal organs. It is likely that 1200 mg/kg BW is a lethal dose (LD_{50}) of CE for the animal model in our study. Similar findings regarding acute toxicity of *Physalis peruviana* L. extract at higher doses have also occurred in guinea pigs, with 800 mg/kg BW shown to be the lethal dose [35]. In our study, a CE dosage of 1200 mg/kg BW resulted in higher expressions of NF-κB and TGF-β1 that similar to IMQ group, but lower Baker and PASI scores. It can be assumed that there was a severe inflammation that may induce signaling pathways that involved in apoptosis program as stated by the previous study [36]. This apoptosis process may relate to pro-inflammatory cells death so that HE staining from CE 1200 mg/kg BW showed remarkable reduced psoriasis changes.

This study is the first to determine that CE from Purwokerto, Central Java, Indonesia, contains physalins B, D, and F as active ingredients and to demonstrate that CE from whole plant parts may improve psoriasis manifestations in animal models by relieving inflammation. The limitation of this study is different time of animal termination, namely, that individuals in the control and IMQ groups were terminated on the 8th day, while members of the other groups were terminated on the 15th day. This time difference may influence the measured parameters at the end of study. In addition, we did not further investigate the cause of death in mice that were not terminated, so we are unable to determine the factors that may have been associated with their mortality.

Conclusions

This study has demonstrated that the administration of CE improves psoriasis-like dermatitis in mice through inhibition of TGF-β1/NF-κβ pathway, which results in reduced Baker's and PASI scores. Based on these findings, we conclude that CE at a dosage of 800 mg/kg BW exerts the optimum psoriasis mouse models refinement without any sign of toxicity, and suggest that CE in dosages <1200 mg/kg BW (and its adjustment dose) be examined in further clinical study as an anti-psoriasis treatment.

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