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Chloroform Extract of *Plumbago zeylanica* Linn. Roots Ameliorates the Epidermal Thickness of Imiquimod-induced Psoriatic Mice through Cell Cycle and Apoptosis

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Abstract

BACKGROUND: Psoriasis vulgaris is a chronic skin disease which is characterized by recurrent scales on the skin. The global prevalence of this disease has increased in 10 years. Plumbagin is an active compound in P. zeylanica Linn. Some recent studies revealed that P. zeylanica Linn. extracts have the antiproliferative activity, which is used for the treatment of some human diseases.

AIM: The aim of this study was to investigate the effect of chloroform extract of P. zeylanica Linn. roots (CEP) on epidermal thickness of imiquimod-induced psoriatic mice.

METHODS: This was a *posttest-only control group design.* A total of 42 male BALB/c mice were divided into six groups. Mice in the treatment groups orally received 25, 50, and 100 mg/kg body weight CEP, respectively, while the positive control (CC, NC, PC and T1-3) orally received 1 mg/kg body weight methotrexate for 7 days. Evaluation of epidermal thickness was based on histological changes, serum IL-23 level by ELISA, and cyclin-dependent kinase 2, cyclin A, and caspase-3 expressions by immunohistochemistry.

RESULTS: Administrations of CEP decreased the epidermal thickness of psoriatic plaques in all treatment groups ($p = 0.002$, 0.003, and 0.016, respectively) compared to negative control but it did not reduce the serum IL-23 level. The expressions of CDK2 and cyclin A reduced in T2 and T3 groups and the expression of caspase-3 increased was only in T3 group.

CONCLUSION: Chloroform extract of *P. zeylanica* Linn. roots administrations reduces the epidermal thickness of imiquimod-induced psoriatic mice by inhibition of keratinocyte cell cycle and induction of caspase-3 expression.

Introduction

Psoriasis vulgaris is a chronic skin disease, which is characterized by recurrent scales in predilection sites [1]. The Global Burden of Disease Study reported that the global prevalence of psoriasis increased by 17.6% during 2005–2015 from 195 countries [2]. So far, limited data is available on the prevalence of *P. vulgaris* in Indonesia. There were two publications from Surabaya, East Java, which stated that 36 psoriasis cases were found during 2016–2017 [3] and 60 cases in Banyumas, Central Java, in 2019 [4].

Etiology of *P. vulgaris* has not been established yet but a recent theory stated that inflammation plays an important role in immune disorders of *P. vulgaris*. Inflammatory cytokines such as tumor necrosis factor (TNF)-α, *interferon gamma* (IFN-γ), interleukin (IL)-23, IL-17, IL-12, IL-21, and IL-22 stimulate the proliferation of epidermal keratinocytes and increase epidermal thickness [1], [5]. Skin trauma and bacterial infections also activate nuclear factor kappa beta (NF-kB) protein through interaction with toll-like receptors protein in myeloid dendritic cells (mDCs), resulting in release of the IL-23 cytokine. High production of IL-23 stimulates hyperproliferation of keratinocytes which is activated by IL-17 activation [6]. In addition, some studies reported that reduction of apoptotic keratinocytes is involved in the etiology of *P. vulgaris* but this process remains controversial. Elango *et al*. study reported that human caspase-3 expression on psoriatic lesions is lower than caspase-3 expression on normal skin of healthy humans [7] while the human caspase-3 expression on psoriatic skin from Bebars *et al.* study (2017) is opposite to the

earlier study [8]. Due to the reduction of keratinocytes apoptosis, the expressions of cyclin-dependent kinase (CDK)-2, cyclin D1, and cyclin E significantly increased on psoriatic skin compared to human normal skin [9], [10].

Plumbago zeylanica Linn. is a tropical plant, which is widely grown in some countries of Asia and Africa continents [11], [12], [13], [14]. Conventionally, *P. zeylanica* Linn. is frequently used for topical application on targeted skins such as ringworm [15]. This plant also consumed orally to treat cancer, leukoderma, and psoriasis [16], [17]. *Maritinone* is a bioactive compound isolated from *ethyl acetate* extract of *P. zeylanica* Linn. roots which had a stronger inhibition of cells proliferation in four cancer cell lines compared to the non-treated cancer cell lines. After 72 h incubation with 5 μM maritinone, the growth of four cancer lines in the G2 and M phases reduced while the cell population increased in the sub-G1 phase [18]. So far, *P. zeylanica* Linn. extracts from leaves, roots, or stems have not been reported for the treatment of chronic skin diseases including psoriasis.

Plumbagin is a homodimer form of *naphthoquinone* with higher concentration in *P. zeylanica* Linn. roots than in the leaves and stems [19]. *In silico* study indicated that plumbagin is able to interact with thymidylate synthase protein better than a cytotoxic agent, 5-fluorouracil [20]. Furthermore, administration of 1.50 μM pure *plumbagin* for 24 h downregulates cyclins (D1 and E1) and CDK-2, -4, and -6 in T24 and UMUC3 human bladder cancer cell lines [21]. Therefore, we investigated the effects of chloroform extract of *P. zeylanica* Linn. roots (CEP) on IL-23 serum level, epidermal thickness, and protein expression of CDK2, cyclin A, and caspase-3 in BALB/c psoriatic mice. Because plumbagin is a naphthoquinone family and has low solubility in water, we extracted *P. zeylanica* Linn. roots using chloroform solvent [22].

Materials and Methods

Preparation of CEP

P. zeylanica Linn. roots were obtained from Palembang, South Sumatera, Indonesia. The plant was authenticated by a botanist and the plants were deposited at the Department of Biology, Faculty of Education, Universitas Muhammadiyah Palembang, South Sumatera, Indonesia. Fresh roots were dried at 25–30°C for 5 days. Dried roots were soaked in lime juice for 48 h and redried at 25–30°C for 3 days [22]. Dried roots were then grounded into powder which macerated in chloroform (Merck) with 1:10 ratio for 24 h. After filtration with Whatman filter paper, the filtrate was concentrated using a rotary evaporator and was kept in a refrigerator until further use. The secondary metabolites contained in our extract were characterized using GC–MS technique [23].

Animal model

The protocol of animal experiments in this study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University/General Hospital Dr. Sardjito, Yogyakarta, Indonesia, number KE/FK/0192/EC/2020 on February 13, 2020. Male BALB/c mice which aged 8–10 weeks old and had 25–35 g body weight (BW) were provided by PT Bio Farma (Bandung, Indonesia). This study was conducted between August and October 2020.

During the experiment, each mouse was housed in a plastic cage with 12 h day and 12 h night cycles and 23°C air temperature. All mice received a standard food and freely accessed tap water. The back skin of mice was shaved with 4x4 cm^2 in size. Five percent (w/w) Imiquimod (IMQ) cream (Aldara, UK) was applied on mice back skin for 4 consecutive days, while Vaseline cream was applied on back skin of the normal control group. In the 5th day induction, one mouse from each group was sacrificed to analyze the thickness of epidermal back skin using the hematoxylin-eosin staining (Figure 1a).

Research design and protocol of research study

The sample size of this study was calculated using the Federer formula for six groups and each group consisted of at least four mice. The selected mice were randomly divided into normal control group (CC), negative control group (NC), positive control group (PC), and treatment groups (T1, T2, and T3).

The NC mice were orally given 5% (v/v) Tween-80 and were intraperitoneally injected with 0.9% sodium chloride every day to prevent dehydration due to IMQ side effect. Meanwhile, the PC mice were orally administered with 1 mg/kg BW methotrexate (MTX) every morning for 7 consecutive days. The mice in the T1, T2, and T3 groups were orally administered 25, 50, and 100 mg/kg BW CEP, respectively, every morning for 7 consecutive days. Due to the low solubility in water, MTX and CEP were dissolved in 5% (v/v) Tween-80. Administration of MTX and CEP began in the 5th day of plaque induction (Figure 1a). To monitor the efficacy of CEP treatment, three mice in each group were sacrificed under ketamine anesthesia in the 8th and 11th days. Cardiac blood samples were collected into the serum tube and back skin tissues were kept in formalin solution for further analysis.

Histopathology and immuno-staining

Formalin-fixed back skin tissues were embedded in paraffin blocks and were then sliced into 5 μm thick sections. The evaluation of tissue histopathology was performed under a light microscope. For histopathology staining, the back skin tissue

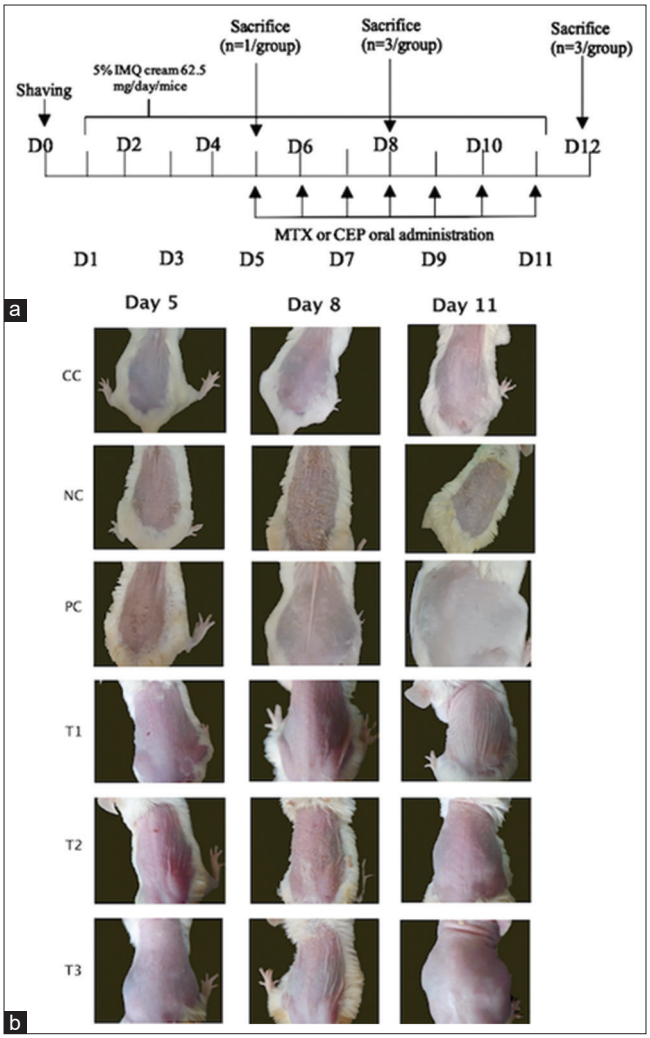

Figure 1: Research protocol and results of CEP administrations. (a) Psoriatic mice were created by application of 5% IMQ cream on the back skin of BALB/c mice for 4 consecutive days and followed by 7 consecutive days during oral administration of MTX or CEP (25, 50, or 100 mg/kg) was administered orally. (b) CEP exerts improvement on IMQ-induced psoriatic mice. Comparison of back skin in each group on day 5, after 3 days of treatment (day 8), and after 7 days of treatments (day 11)

sections were stained routinely with hematoxylin-eosin and were evaluated by a pathologist to determine epidermal thickness. In brief, immunohistochemical staining was carried out using antibodies directed against the antigen caspase-3 (Bioss, USA, catalogue no. bs-2593R), CDK2 (Bioenzy, Indonesia, catalogue no. BZ-0872610F-AP), and cyclin A (Bioenzy, Indonesia, catalogue no. BZ-0814320F-AP), according to the manufacturer's instructions. Evaluation of caspase-3, CDK2, and cyclin A expressions was performed by two independent pathologists. Histopathologic scoring was done using a formula: Intensity grade (none, weak, moderate, or strong) x percentage of stained cells.

Enzyme-linked immunosorbent assay (ELISA)

Interleukin-23 mouse serum was measured using a specific antibody from Abbexa, UK (catalogue no. abx254193), according to the manufacturer's protocol. The mean of IL-23 serum level of mice in all groups was presented in pg/ml.

Statistical analysis

All statistical analyses were done using the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Epidermal thickness of back skin and IL-23 serum level were presented as the mean ± SD, respectively. The mean differences of epidermal thickness and IL-23 serum level among groups were analyzed using the one-way ANOVA test and followed by the LSD multiple comparison test. The mean differences between the 8th and 11th days within groups were analyzed using the paired t-test with $p < 0.05$ for significant consideration.

Originally, the data of histopathologic scores of cyclin A, CDK2, and caspase-3 expressions were categorized as ordinal data which were then converted into numbers (0, 1, 2, and 3) and multiplied by the percentage of stained cells. Histopathological scores of caspase-3, CDK2, and cyclin A were expressed in median and the median differences among groups were analyzed using the Kruskal–Wallis test. Meanwhile, the median differences between the 8th and 11th days within groups were analyzed using the Wilcoxon test with p <0.05 for significant consideration.

Results and Discussion

Oral administration of CEP reduced epidermal thickness in psoriatic mice induced by 5% IMQ cream

To investigate whether CEP had an advantageous effect on epidermal thickness or not, we administered CEP with three different doses to psoriatic mice induced by 5% IMQ cream (Figure 1b). After 5 days erythema appeared on mice back skin. After MTX and CEP oral administrations, the erythema in four treatment groups reduced in day 8, compared to NC group. Meanwhile, the reduction of erythema and squama clearly occurred in day 11 but only psoriatic mice in the PC and T3 groups had similar appearance of back skin to the CC group.

Mouse epidermis consists of four layers, that are, stratum corneum, granulosum, spinosum, and germinativum (Figure 2a). We evaluated the epidermal thickness of back skin after CEP administration. We found that IMQ-treated skin lesions displayed augmented the epidermal hyperplasia (Figure 2b) and administration of MTX and CEP significantly reduced the epidermal thickness of psoriatic mice (Figure 2c).

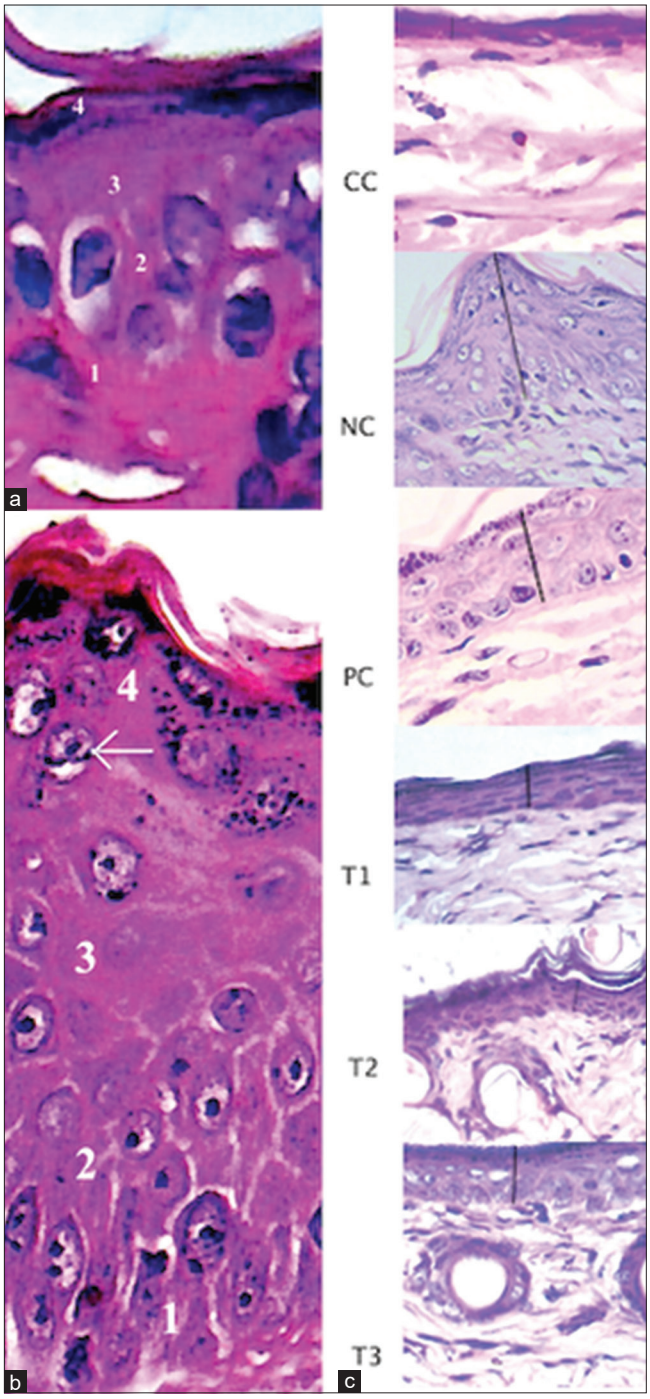

Figure 2: Hematoxylin and eosin staining of mice back skin (×400). (a) Epidermal layers of CC group in day 5 consisted of 1 = Stratum basal, 2 = Stratum spinosum, 3 = Stratum granulosum, and 4 = Stratum corneum. (b) Epidermal layers of PC group in day 5 consisted of 1 = Stratum basal, 2 = Stratum spinosum, 3 = Stratum granulosum, and 4 = Stratum corneum, black arrow indicated parakeratosis. (c) Epidermal thickness of all groups (black line)

Measurement of epidermal thickness was used to confirm the macroscopic and microscopic appearances of psoriatic mice treated with either MTX or CEP oral administrations (Figure 3a). On day 8, psoriatic mice in PC group had the highest average of epidermal thickness and followed by the T1 group, while the NC and T3 groups had similar epidermal thickness. The lowest epidermal thickness was observed in thT2 group. The epidermal thickness in those groups significantly differed from the epidermal thickness in the CC group (p = 0.009). However, the epidermal thickness in all treatment groups did not differ from the NC group. After 7 days treatment (day 11), the average of epidermal thickness in the CC and NC groups increased while the average of epidermal thickness in other groups decreased. In comparison to the NC group, the average of epidermal thickness reduced significantly in the PC (p = 0.007), T1 (p = 0.002), T2 (p = 0.003), and T3 (p = 0.016) groups.

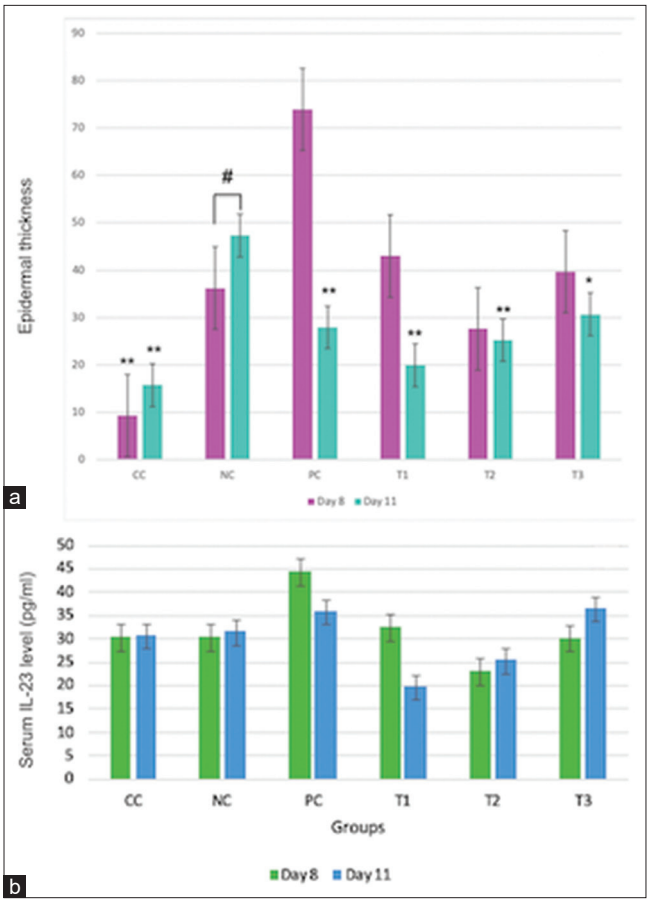

Figure 3: Epidermal thickness and serum IL-23 level. (a) Epidermal thickness of back skin (μm) in day 8 and day 11 in each group. Data were expressed as the mean ± SEM. One-way ANOVA showed p < 0.05 (*) and p < 0.01 (**) versus mice in the NC group. Paired t-test showed p < 0.05 (#) within NC group. (b) Serum IL-23 level (pg/ml) in day 8 (green bar) and day 11 (blue bar) in each group showed no difference versus serum IL-23 in the NC group. Data were expressed as the mean ± SD

Interleukin-23 serum level in psoriatic mice remained stable after CEP oral administrations

Because IL-23 plays a key role in the pathogenesis of psoriasis, we assessed the IL-23 serum level using the ELISA method. In Figure 3b, each group had different levels of serum IL-23 either in day 8 or in day 11 except the CC group. In day 8 intervention, the PC group showed the highest level of serum IL-23 and followed by the T1 group. The average of serum IL-23 level was similar in the CC, NC, and T3 groups while the lowest level of serum IL-23 was found in the

T2 group. In the end of the intervention, the serum IL-23 levels decreased in the PC and T1 groups while increased in the NC, T2 and T3 groups. However, the different levels of serum IL-23 among groups did not show significantly.

Oral administration of CEP inhibited keratinocyte cell cycle in epidermis of psoriatic mice

The expression of CDK2 and cyclin A proteins is important biomarkers for evaluation of cell cycle. Immunohistochemical staining showed the expressions of CDK2- and cyclin A-positive cells in the epidermis of mice back skin (Figure 4a). The immunostaining intensity in the PC group was similar to the NC group while the T1, T2, and T3 groups had lower immunostaining intensity than the NC group. In addition, the CC group had the lowest immunostaining intensity compared to other groups.

Further analysis of CDK2 and cyclin A histopathologic scores indicated that CEP oral administrations had different effects. Lower CDK2 histopathologic scores were observed in the PC, T2, and T3 groups in day 11 compared to the CDK2 histopathologic scores in day 8, including the CC group. Meanwhile, the CDK2 histopathologic scores in the NC and T1 groups increased (Figure 4b).

In contrast to CDK2 histopathologic scores, cyclin A histopathologic scores increased in the CC, PC, and T1 groups in day 11 versus day 8 intervention while the cyclin A histopathologic scores in the NC, T2, and T3 groups decreased (Figure 4c). Overall, the histopathologic scores of CDK2 and cyclin A did not significantly differ from all groups.

Oral administration of CEP augmented the caspase-3 expression in epidermis of psoriatic mice

Caspase-3 protein is a well-known biomarker for the evaluation of cellular apoptosis. We investigated the caspase-3 expression in psoriatic mice treated with CEP. Immunostaining intensity in PC group was higher than the immunostaining intensity of other groups (Figure 5a). From histopathologic scores (Figure 5b), caspase-3 expressions in the CC and T3 groups increased while caspase-3 expressions in other groups decreased from day 8 to day 11 intervention. The statistical analysis of caspase-3 histopathologic scores indicated no differences among all groups.

In the present study, we hypothesized that CEP administrations could ameliorate epidermal thickness of psoriatic plaques in IMQ-induced psoriatic mice through inhibition of keratinocyte S phase cell cycle and upregulation of the intrinsic apoptotic

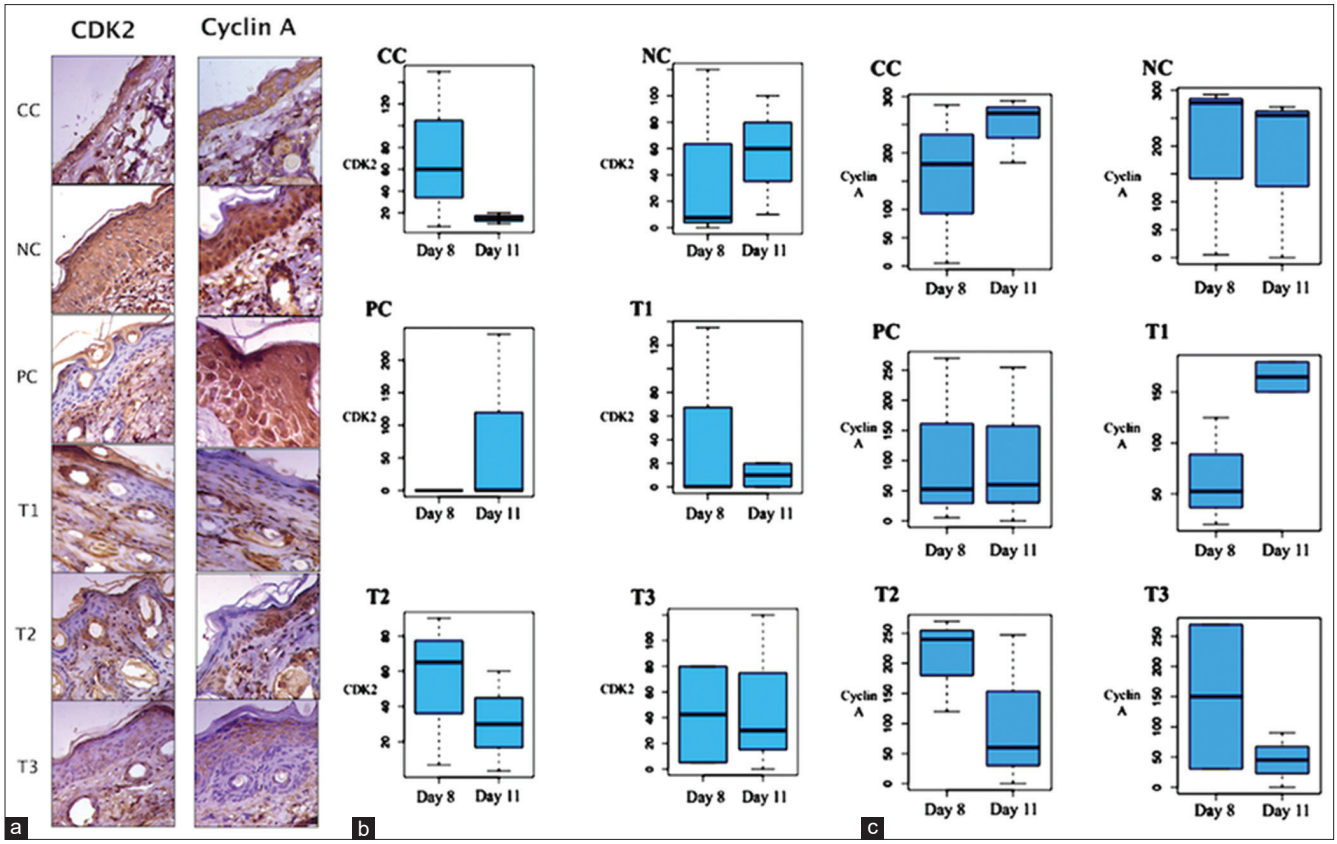

Figure 4: CEP exerts improvement on epidermal thickness of treated mice (T1, T2, and T3) compared to untreated mice (NC). (a) IHC staining (×400) showed downregulated expression of CDK2 and cyclin A in the T1 and T2 groups. (b) Expression of CDK2 in plaque using immunohistochemistry technique. Data were presented in median using the boxplot. (c) Cyclin A expressions in plaque using immunohistochemistry technique. Data were presented in median using the boxplot

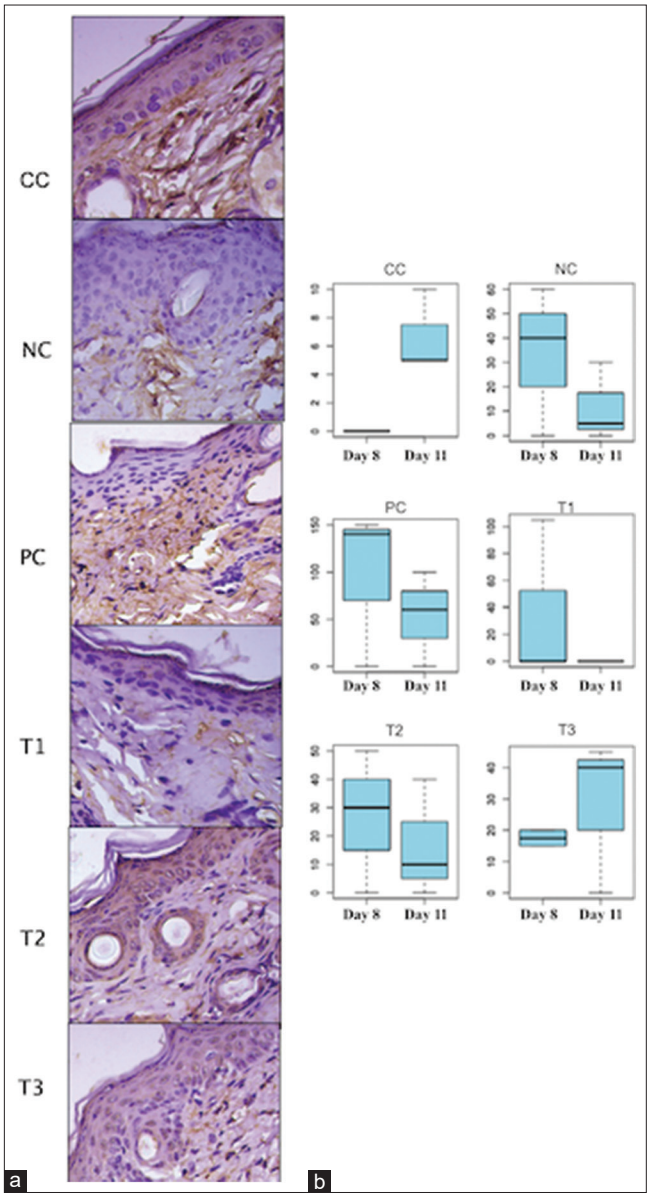

Figure 5: (a) IHC staining (×400) showed upregulated expression of caspase-3 in the PC, T2, and T3 groups compared to the NC group. (b) Histopathologic scores of caspase-3 in skin plaque lesions were calculated by multiplication of IHC staining score and positive stained cells. Data were presented in median using the boxplot

pathway. Our results showed that the administration of 50 and 100 mg/kg BW CEP ameliorated the epidermal thickness due to inhibition of CDK2 expression, similar to 1 mg/kg BW MTX administration. The cyclin A expressions decreased in psoriatic mice treated with 50 and 100 mg/kg BW CEP, while cyclin A expressions increased in psoriatic mice treated with 1 mg/kg BW MTX. The highest dose of CEP administration upregulated the caspase-3 expressions, while other doses of CEP and MTX administrations downregulated the caspase-3 expressions. However, we observed stable IL-23 serum levels throughout the experiment in all psoriatic mice groups.

Psoriatic mice model with IMQ induction, which is characterized by NF-kB release from mDC and activation of IL-23, is frequently used to study the pathogenesis of human psoriasis and to develop new drugs such as herbal medicines. Our finding indicated that IL-23 serum levels remain stable during the CEP intervention. This result is consistent with the previous research study, which stated that there was no significant difference in serum IL-23 levels between the control and genetically engineered K23 psoriatic arthritis mice groups [24]. In contrast, higher serum IL-23 levels [25], [26], [27] and plasma IL-23 levels [28] were observed in IMQ-induced psoriatic mice compared to the normal mice. Those different results may be due to the different time of blood sampling. In our study, we took the blood sampling in day 8 and day 11 after IMQ induction, while Takuathung *et al.* (2018) took the blood samples in day 17 after IMQ induction [25]. In addition, Wang *et al.* (2019) and Hao *et al.* (2021) performed the blood sampling similar to our study in day 7 and day 9, respectively [26], [27]. From gene expression viewpoint, IL-23 mRNA in the psoriatic plaque increased after ±2 days exposure of 5% IMQ cream and then decreased almost zero after ± 4 days [29]. On the other hand, other studies reported that IL-23 mRNA expression increased after 4 days induction with 5% IMQ cream in psoriatic mice [30], [31], [32]. Meanwhile in the protein expression level, IL-23 in Swiss Albino mice with psoriatic plaque was significantly higher than that of control mice [33].

Hyperproliferation of the keratinocytes due to high production of IL-23 is the main clinical manifestation of *P. vulgaris*, resulting in upregulation of cell cycles and downregulation of apoptotic cells. In our knowledge, there is a limited publication related to the cell cycle on IMQ-induced psoriatic plaques. Upregulation of cyclin B2 [34] and cyclin D1 expressions [27], [35] was found in psoriatic mice treated with 5% IMQ cream, compared to the control mice. In our findings, mice treated with 50 and 100 mg/kg BW CEP had lower cyclin A and CDK2 expressions than the control group. Theoretically, the CDK enzymes will become active when interacts with cyclins to form cyclin-CDK complexes, including cyclin A-CDK2 complexes to proceed the S phase during cell cycle. Our result supports a previous *in vitro* study that pure plumbagin downregulated CDK2 expression in human bladder cancer cell lines [21]. Moreover, CDK2 expression on human psoriatic plaques is higher than that of healthy control [10]. Therefore, our study provides important data related to inhibition of cell cycle using CEP in IMQ-induced psoriatic mice, resulting in reduction of keratinocyte hyperproliferation.

From intrinsic apoptosis pathway, DNA damage will activate caspase-2 and caspase-9 proteins as the apoptosis effector, leading to the activation of caspase-3 as a common indicator of cell apoptosis. Administration of 100 mg/kgBW CEP in IMQ-induced psoriatic mice increased caspase-3 expression while administrations of 50 mg/kgBW CEP and 1 mg/kgBW MTX decreased caspase-3 expression. These results differ from Duan *et al.* (2020) study that intradermal administration of

1.5 mg/kgBW receptor interacting protein kinase-1 R-7-Cl-O-Necrostatin-1 did not increase the caspase-3 expression in IMQ-induced psoriatic mice but only reduced cell necroptosis [36]. Overall, it seems that administration of the highest CEP dose containing plumbagin inhibits cell cycle and induces apoptosis in psoriatic plaques but a further investigation is required to figure out its action mechanism of plumbagin.

Downregulation of the cyclin A-CDK2 complexes and upregulation of the Caspase-3 expression during CEP administrations are dose-dependent manner. However, the important findings in our study still have some limitations. At first, serum IL-23 levels vary between treated and untreated mice so that this cytokine is not a good biomarker for the evaluation of psoriasis therapy. We also evaluated cell cycle and apoptosis using the immunohistochemical technique to measure cyclin A, CDK2, and caspase-3 expressions but the results are not consistent between the negative control, positive control, and treatment groups in psoriatic mice.

Conclusion

Chloroform extract of *P. zeylanica* Linn. roots containing plumbagin reduces the epidermal thickness of IMQ-induced psoriatic mice by inhibition of keratinocyte cell cycle and induction of keratinocyte apoptosis.

For quantification of Cyclin A, CDK2, and Caspase-3 expressions in future, we consider using the ELISA kit. In order to distinguish the beneficial effect of CEP administrations, we need to apply a combination of CEP and MTX for longer time.

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