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Research Article

Anti-inflammatory potential of Indonesian plants in human keratinocytes

Tae-Young Gil[1], Bo-Ram Jin[1], Yea-Jin Park[1,2], Hyo-Jung Kim[1], Divina C. Cominguez[2], Hye-Min Kim[2], Hee-Young Kim[3], Agung Nugroho[4], Hyo-Jin An[1,3], and Kyou-Young Lee[5,*]

[1] Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea
[2] Department of Rehabilitative Medicine of Korean Medicine and Neuropsychiatry, College of Korean Medicine, Sangji University, Wonju, Gangwon-do 26339, Republic of Korea
[3] Department of Integrated Drug Development and Natural Products, Graduate School, Kyung Hee University, Seoul 02447, Republic of Korea
[4] Department of Agro-industrial Technology, Lambung Mangkurat University, Banjarbaru, Indonesia
[5] Department of Korean Ophthalmology and Otolaryngology and Dermatology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do 26339, Republic of Korea.
*Correspondence: lkyy0706@sangji.ac.kr (K.-Y.L.); Tel.: +82-33-730-8075

Abstract: A variety of Indonesian plants show different medicinal effects as traditional herbal medicines. Since severe inflammation would lead chronic diseases, regulating inflammatory reactions on time is crucial for protection against worsen conditions. High effectiveness is important as much as easy accessibility and popularity such as local plants named folk medicines. This study shows the effect of commonly using three plants extracts in Indonesia. *Carica papaya*, *Stenchlaena palustris*, and *Euphorbia hirta* were extracted using different solvents which are various percentage of methanol, distilled water, or ethanol. The extracts were examined available concentrations without cell toxicity in human keratinocytes, HaCaT. Also, the effects on pro-inflammatory cytokine, interleukin-6, production were evaluated in the cell using enzyme-linked immunosorbent assay. The results present the potential of Indonesian herbs extracts as down-regulating agents for inflammation.

Keywords: folk medicine; Indonesian plants; inflammation; HaCaT keratinocytes

1. Introduction

Inhibiting inflammatory reactions could be an essential step for regulating and preventing many severe chronic diseases (Furman et al., 2019). It implies the importance of anti-inflammatory therapeutic agent discovery. It has been widely known that a plentitude of herbal extracts or their compounds work as medicines for a long time (Grossmann 1957). Especially, plant extracts have been used for various diseases with high accessibility with no needs for particular formulating techniques (Ameer et al., 2017). Besides, plant extracts include bioactive soluble or insoluble ingredients following the characteristics of solvents which have considered synergetic effects compared to single compounds (Proestos 2020). Also, extracts are accessible as not only medicine but also daily diet with high accessibility (Pagano, et al., 2021). Given these advantages of extracts, a screening test is conducted to evaluate the toxicity or regulating pro-inflammatory cytokine production in immortalized human keratinocytes.

Keratinocytes are composed about more than 90 % of epidermal cells playing crucial roles in skin health (Gil et al., 2022). Since skin acts the outermost and largest organ protecting one's body against harmful matters or microorganisms, keeping healthy skin would mean supporting the body immune system (Nasiri et al., 2020). Physically, skin is the first line against the allergen, bacteria, toxins, or any other causes: internal first response to infection or irritation is inflammatory reaction (Vaknin and Baniyash 2011). Therefore, we applied immortalized human keratinocytes to evaluate the effects of Indonesian plants extracts on the major pro-inflammatory cytokine, interleukin-6 (IL-6). Stimulated keratinocytes with tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) induce a variety of pro-inflammatory genes including C-X-C Motif Chemokine Receptor (CXCR) or ILs (Piipponen et al., 2020). Among different cytokines or chemokines, IL-6 plays a critical role in the acute phase responding to a various clinical and biological features at the site of inflammation (Gabay 2006). In this paper, three Indonesian plants extracts were used for

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determining the effects on IL-6 production; *Carica papaya* (*C. papaya*), *Stenchlaena palustris* (*S. palustris*), and *Euphorbia hirta* (*E. hirta*).

In the almost entirely tropical climate of Indonesia, characteristic species of plants have been grown such as *C.papaya* (Hanny 2013). Its fruit is well known for the sweetness and nutrients such as powerful antioxidant vitamins against various symptoms including cancer, inflammation, aging, or skin disease (Aravind et al., 2013; Kong et al., 2021). *S. palustris* is dietary medicinal species used for fever, skin disease, ulcerative colitis, or stomach (Benjamin and Manickam 2007). *E. hirta* is easily found weed treating ulcers, dysentery, gastrointestinal or skin infections, diabetes mellitus or respiratory disorders (Silalahi 2021). These plants extracts were evaluated the available concentration with no cell toxicity and regulating effects on the pro-inflammatory cytokine production in HaCaT cells.

2. Materials and Methods

2.1. Materials and Reagents

Dulbecco's modified Eagle's medium (DMEM), inactivated fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Life Technologies Inc. (Grand Island, NY, USA), Gibco, or HyClone. Dimethyl sulfoxide (DMSO) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were supplied by Sigma Aldrich (St. Louis, MO, USA), Wako chemical Co. Ltd (Tokyo, Japan).

2.2. Preparation for extraction

All the plant extract materials were kindly provided from professor Agung Nugroho. Leaves of *C. papaya* were collected from a papaya farm near Pelaihari City, South Kalimantan Province, Indonesia. Aerial parts of *E. hirta* or *S. palustris* were gathered in Banjarbaru, South Kalimantan Province, Indonesia. The plant material dried in a dark room at room temperature for four days. To obtain dried materials with constant weight, the airy dried samples were continually dried using an oven dryer at 40 ℃ for 12 h. Then, extraction under reflux was employed following the extraction method following the previous described by Nugroho et al (Nugroho, Heryani et al. 2017, Nugroho, Heryani et al. 2020). 500 g of each dried plant part were extracted using 5 L of various solvents for 5 h at 70 ℃. After extraction, the solution was filtered with filter paper and evaporated under vacuum using a rotary evaporator. Evaporation was performed until there was no solvent evaporated and condensed. And then, a semi-solid state of the extract conducted. The obtained extract was weighed to measure the yield percentage of each sample which is detailed in the previous studies. The enriched sample was lyophilized, and a yield of 62 g CPE was obtained. Plant partial yield ranges are written following; leaves of *E. hirta* (24.88 ± 0.71 mg/g), flowers (15.17 ± 0.45 mg/g), and stems (15.65 ± 0.46 mg/g).

2.3. Cell culture and plant extract treatment

HaCaT keratinocytes were cultured at 37 °C in DMEM supplemented with 10% inactivated FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified atmosphere with 5% CO2. Cells were pre-treated with Indonesian plants extracts at different concentrations, and then stimulated using a mixture of TNF-α and IFN-γ (each 10 ng/mL) for inducing inflammatory reactions.

2.4. Cell viability assay

Cell viability was determined using the colorimetric MTT assay. Briefly, Indonesian plants extracts-treated cells were incubated for 24h. Next, the cells were kept in 5 mg/mL MTT solution for 4 h at 37°C. DMSO was used to dissolve the insoluble formazan product after removal of the supernatant. Cell viability was measured at 570 nm using an Epoch microplate spectrometer (BioTek, Winooski, VT, USA). Experiments were conducted in thrice in parallel for each concentration of Indonesian plants extracts, and the results were expressed as the mean ± standard error mean (SEM).

2.5. IL-6 analysis

Culture media were obtained approximately 24 h after treatment with Indonesian plants extracts and stored at −70 ◦C. The levels of IL-6 were assessed using enzyme immunoassay (EIA) kits for humans (BD OptEIA™, BD Science, San Jose, CA, USA) according to the manufacturer's instructions.

2.6. Statistical analysis

Data were expressed as the mean ± SEM of three experiments. Comparisons among groups were performed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Indonesian plants extracts were showed cell viability in some concentrations in immortalized human keratinocytes.

C. papaya leaves extracted with different solvents such as 100% methanol (MeOH) (CPE100M, Fig. 1A), 50% MeOH (CPE50M Fig. 1B), or distilled water (DW) (CPEDW, Fig. 1C). Different solvent would be applied to make higher induction of various characteristic solubility and ingredients. CPE100M showed cell toxicity in the concentrations; 80% cell viability at 62.5 μg/mL (Fig. 1A). CPE50M showed more than 80% of cell viability at all concentrations in HaCaT cells. Minimal cell viability is 80.37% at 62.5 μg/mL of CPE50M (Fig. 1B). CPEDW which was used distilled water for extract C. papaya presented cell toxicity more than 62.5 μg/mL of the extracts showing lower cell viability than 78% (Fig. 1C). Also, the authors examined the cell viability with another Indonesian plant, *S. paulustris* leaves, extract (SPE) which was extracted with water (SPEDW, Fig. 1D) or 30% EtOH (SPE30E, Fig. 1E). SPEDW showed cell toxicity (lower than 80% cell viability) at the concentrations more than 31.25 μg/mL (Fig. 1D). SPE30E showed higher toxicity at the concentration more than 15.625 μg/mL with lower cell viability than 80% (Fig. 1E). These results showed the different solvent to extract Indonesian plants had different cell toxicity in human keratinocytes.

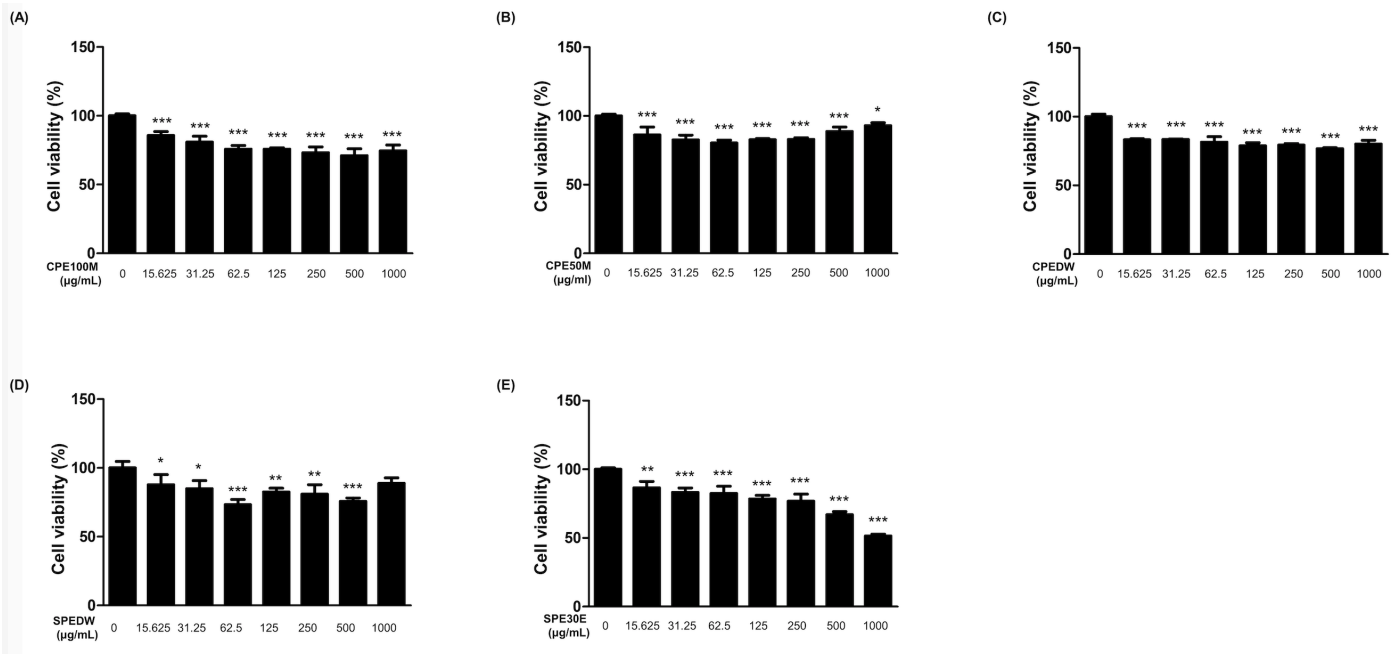

Figure 1. Effects of Indonesian plants extracts on HaCaT keratinocytes viability. Human keratinocytes HaCaT cells were treated with the indicated amount of Indonesian plants extracts for 24 hr and then cell viability was determined by MTT assay. Data were the mean ± SEM. *p<0.05, **p<0.01, and ***p<0.001 versus none-treated group (concentration as 0 μg/mL) by ANOVA and Dunnett's post-hoc test.

3.2. Different Indonesian plants extracts showed various results on the pro-inflammatory cytokine production.

TNF-α and IFN-γ were used to stimulated the immortalized human keratinocytes mimicking skin inflammation in vivo inducing inflammatory circumstance (Piipponen, Li et al. 2020). With the results for cell viable concentrations of extracts (Fig. 1), the authors selected different concentrations for further study. TNF-α and IFN-γ treatment led the significant increase of IL-6 production compared to the non-treated group. CPE100M (15 and 30 μg/mL), SPEDW (15 and 30 μg/mL), and CPE30E (7 and 14 μg/mL) showed no any changes on the IL-6 production under TNF- α and IFN-γ

stimulations (Fig. 2A, 2D, and 2E). CPE50M treatment showed different tendency compared to the TNF-α and IFN-γ stimulated group which were decrease at 500 μg/mL of CPE50M as well as increased IL-6 production at 1000 μg/mL of treatment (Fig. 2B). 30 μg/mL of CPEDW treatment showed increased IL-6 production and 60 μg/mL of CPEDW treatment could decrease on the production (Fig. 2C).

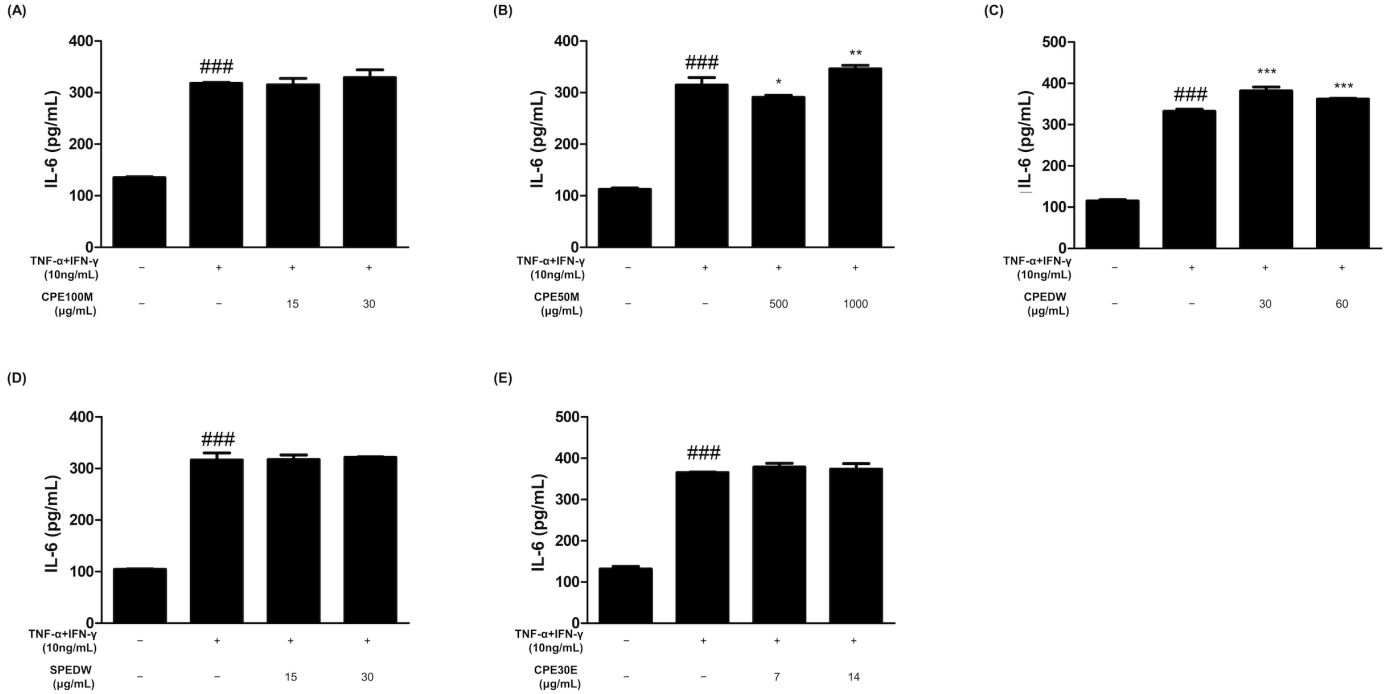

Figure 2. Effects of Indonesian plants extracts on inflammatory cytokine production in HaCaT keratinocytes Cytokine production was evaluated with ELISA. Cells were treated with the indicated amount of Indonesian plants extracts for 1 hr prior to additional stimulation of TNF-α and IFN-γ and then incubated for 24 hr. Data were presented as the mean ± SEM. *p<0.05, **p<0.01, and ***p<0.001 versus none-treated group (concentration as 0 μg/mL), ###p<0.001 versus the TNF-α and IFN-γ treated group; statistical significance of differences between the groups was evaluated by ANOVA and Dunnett's post-hoc test.

3.3. Partial extracts of E. hirta showed different cell viability in immortalized human keratinocytes.

E. hirta extracts were prepared with different parts; leaves, flower, or stems whose cell viability results were shown in figure 3 (A-C). The leaves extract (EHE-L) showed cell viability up to 250 μg/mL with 82% (Fig. 3A). *E. hirta* flower extract (EHE-F) presented cell viability (80%) at the concentration lower than 62.5 μg/mL (Fig. 3B). Its extract from stems was shown cell viability at the all concentrations (Fig. 3C).

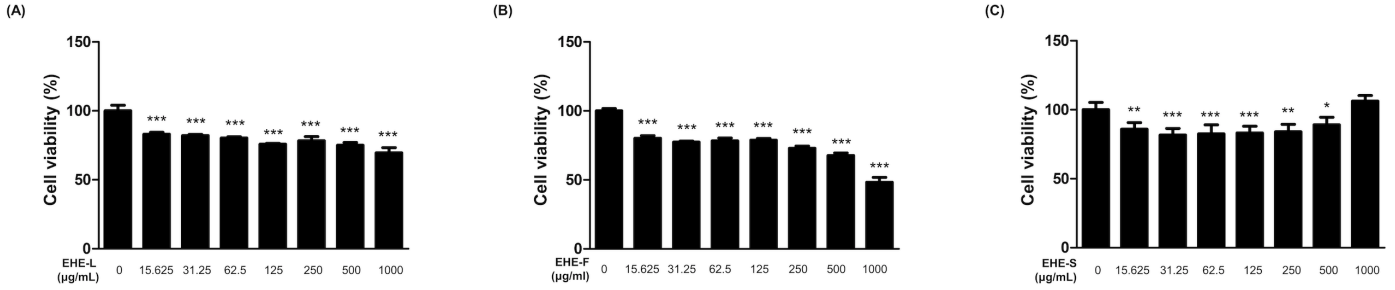

Figure 3. Effects of Indonesian plants' partial extracts on HaCaT keratinocytes viability Human keratinocytes HaCaT cells were treated with the indicated amount of partial *E. hirta* extracts for 24 hr and then cell viability was determined by MTT assay. Data were the mean ± SEM. *p<0.05, **p<0.01, and ***p<0.001 versus none-treated group (concentration as 0 μg/mL) by ANOVA and Dunnett's post-hoc test.

3.4. Leaves extracts of E. hirta showed anti-inflammatory potential on the pro-inflammatory cytokine production.

Given the cell viability results in figure 3, the authors decided to use the same concentrations of partial extracts to investigate the differences between the partial extractions – 30 and 60 μg/mL of each extraction concentrations. TNF-α and IFN-γ treatment group showed significant increased IL-6 production. However, only EHE-L could show the decrease pro-inflammatory production in the dependent manner (Fig. 4A). Extractions from flower (EHE-F) or stems (EHE-S) showed no differences compared to the TNF-α and IFN-γ stimulated group (Fig. 4B and 4C).

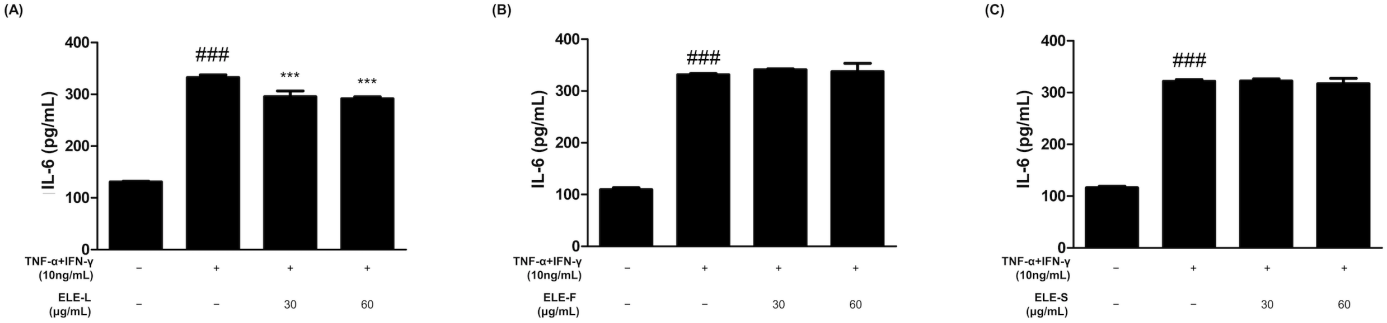

Figure 4. Effects of Indonesian plants' partial extracts on inflammatory cytokine production in HaCaT keratinocytes Cytokine production was evaluated with ELISA. Cells were treated with the indicated amount of *E.hirta* partial extracts for 1 hr prior to additional stimulation of TNF-α and IFN-γ and then incubated for 24 hr. Data were presented as the mean ± SEM. ***p<0.001 versus none-treated group (concentration as 0 μg/mL), ###p<0.001 versus the TNF-α and IFN-γ treated group; statistical significance of differences between the groups was evaluated by ANOVA and Dunnett's post-hoc test.

4. Discussion

This work has shown the potential of several Indonesian plants on the regulating skin inflammation using in vitro model, the immortalized human keratinocytes, HaCaT cells. The plants were three commonly used medicinal ones in Indonesia where has tropical climate; *C. papaya*, *S. palustris*, and *E. hirta.* These specific drugs were selected from among the many available in Indonesia showing anti-inflammatory effects based on their own folk medical usages, previous results, and recommendation by professor A. Nugroho (Nugroho et al., 2017). With the previous studies, *C. papaya* and *S. palustris* used methanol and ethanol for extraction, respectively (Bridge et al., 2015; Asghar et al., 2016; Jafari et al., 2017).

C. papaya is usually famous for its sweet flavor fruit, papaya, and abundant vitamins (Leitao et al., 2022). Not only the fruit, do its leave have the medicinal effects and it has been used for more diverse therapeutic aspects. And the leaves were the more popular material for treating diseases in ethno-pharmacological usages as papaya leaf juice (Hariono et al., 2021; Teh et al., 2022). Anti-inflammation, immuno-modulating properties, anti-cancer and anti-proliferation or apoptosis were shown in the previous studies (Nguyen et al., 2013, Singh et al., 2020; Kong et al., 2021; Ramakrishnan et al., 2021). Also, with the present study results, different extracting solvent showed the different cell viability at the various concentrations (Fig. 1A-C). Following the previous results which have shown the effective solvent The extracts, CPE100M, CPE50M and CPEDW were the different effects on the pro-inflammatory cytokine production in figure 2A-2C. CPEDW was the only one which was able to down-regulate the production under the TNF-α and IFN-γ-treated group mimicking inflammatory condition. It seems to be caused by the different solubility in the various solvent.

S. palustris has a few research results with molecular identification compared to popular folk usages (Kachhiyapatel et al., 2016). It might be caused the lower efficacy with relatively high toxicity seen in figure 1D-1E and figure 2D-2E. Though SPEDW and SPE30E showed high cell toxicity in the concentrations more than 62.5 μg/mL (Fig. 1D and 1E), the effects on the pro-inflammatory cytokine production were not significant at all (Fig. 2D and 2E). It seems to be necessary to perform the assays under the different models avoiding the hasty generalization.

As a medicinal plant (Zhang et al., 2022), *E. hirta* has the plentiful lignans and triterpens (Ragasa and Cornelio 2013). Diverse bio-active compounds in the plant leads the various medicinal effects including anti-bacterial activity, treating diarrhea and constipation (Ali et al., 2020; Ramachandran et al., 2022). These characteristics are considered to be

similarity in the same genus, Euphorbia, which has the Korean medicine, *Euphorbia kansui* (甘遂) (Shen et al., 2016). For this kind of similarity, investigation on the folk medicinal plants could be another chance to develop and discover new medicines.

This screening results of some Indonesian plants on the pro-inflammatory cytokine production could not show the definite guarantee on the anti-inflammatory effectiveness of extracts with the limited detection on one marker, IL-6. However, it could be the initial step for the discovery and development of the folk medicinal plants at least. Since IL-6 plays a crucial role in the acute phase response at the inflammatory site and rules the transitions from acute to chronic inflammation by leading the switch of leucocytes' characteristics (Gabay 2006). In addition, IL-6 is known for a pro-inflammatory cytokine convolutely associated with skin healing as well as inflammation (Lee et al., 2013) which were the interesting pathological conditions. Given the selectable results in the present study, we would like to suggest the potent regulating agents to skin inflammation for further study.

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Conflicts of Interest: The authors declare no conflict of interest.

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