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**Immunomodulatory effect of hexane extract of *Vernonia cinerea* Less. trunk on human peripheral blood mononuclear cells**

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**ABSTRACT**

*Vernonia cinerea* Less. (Asteraceae) or Little ironweed has been demonstrated to possess not only antipyretic and anti-inflammatory effects, but also contain therapeutic activities for alleviating certain gastrointestinal disorders and skin disorders. Trunk is a part of this plant, which is also used as a traditional medicine, but there are no scientific data on anti-inflammatory property in humans. Herein, the present study aimed at evaluating the toxicity of the hexane extract of *Vernonia cinerea* L. trunk and its anti-inflammatory effect on peripheral blood mononuclear cells (PBMCs) of healthy individuals. Using the MTT assay, we found that the hexane extract did not influence cell viability of PBMCs, and the percentage of viable cells observed was more than 90% with regard to treatment with the concentration range of 0.19 to 100 µg/mL of hexane extract. As experimentally tested using an ELISA method, we found that the hexane extract reduced pro-inflammatory cytokine level (IL-6) significantly ( $P < 0.05$ ) when treating the PBMCs with the concentration range of 12.5 to 100 µg/mL, and this decrease in IL-6 might be due to an inhibition of NF-κB nuclear translocation as proven using immunocytochemistry and confocal microscope. Nevertheless, no changes in TNF-α and IL-10 levels were observed. Therefore, this study suggests that the hexane extract of *Vernonia cinerea* L. trunk possesses an immunomodulatory effect on human PBMCs.

**Keywords:** *Vernonia cinerea* Less. trunk, Hexane extract, Immunomodulatory, NF-κB, Peripheral blood mononuclear cells

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## INTRODUCTION

Inflammation is the immune process in response to the invasion of pathogens, and this particular process is influenced by the variety types of white blood cells such as lymphocytes, monocytes, neutrophils and macrophages including the secretion of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 and IL-6, which lead to the migration of leukocytes to the site of infection [1]. Pro-inflammatory cytokines can not only increase the expression of adhesion molecules and vascular permeability, but also induce the chemokine secretion from endothelial cells [2]. NF- $\kappa$ B is one of the most important transcription factors for the secretion of pro-inflammatory cytokines [3]. In the infection location, white blood cells also secrete anti-inflammatory cytokines such as IL-10 and TGF- $\beta$ . Clinically, inflammations lead to many chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, atherosclerosis and autoimmune diseases [4]. All of these disorders, which are needed long-term treatment, are commonly found in Thais and other populations worldwide. We have been interested in herbs that are not only popularly present in Thailand, but also have many therapeutic uses for health improvement. In particular, *Vernonia cinerea* Less. (Asteraceae), also known as Little ironweed, is a kind of plant that possesses numerous medicinal and therapeutic properties. For instance, its leaves or entire plants were used to treat various gastrointestinal disorders, pain and fever, whereas its trunks were used for wound healing and skin disorders [5].

Although many people have used *Vernonia cinerea* L. in the traditional medicine for a long time, there are no scientific data in anti-inflammatory in humans. In the previous study, there was a report that methanolic, chloroform and ether extracts of *Vernonia cinerea* L. leaf possessed analgesic, antipyretic, anti-inflammatory effects in mice [6]. Furthermore, anti-inflammatory effect of an ethanolic extract from the flower of *Vernonia cinerea* L. in adjuvant arthritic rats was demonstrated [7]. Another study reported that methanolic extract could increase levels of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase in blood and liver of carrageenan induced paw edema mice [8]. Recently, methanolic extract reduced levels of nitric oxide (NO) and pro-inflammatory cytokines, and this particular extract acted by downregulating the cyclooxygenase-2 (COX-2) mRNA expression in LPS-stimulated macrophages [9]. Nowadays, no scientific data concerning either its trunk or hexane extract is available. In the present study, we were interested to study immunomodulatory effect of hexane extract of *Vernonia Cinerea* L. trunk on human peripheral blood mononuclear cells (PBMCs) by evaluating its toxicity and studying its anti-inflammatory effect.

## EXPERIMENTAL SECTION

### Preparation of plant extract

*Vernonia cinerea* L. was collected from Kamphaengphet province, Thailand and was authenticated by Professor Dr. Thaweesakdi Boonkerd (Department of Botany, Faculty of Science, Chulalongkorn University, Thailand). The voucher specimen of *Vernonia cinerea* L. was 013426 (BCU), which was deposited at the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. The plant was cut into small pieces and air – dried for 3 days. The extract was prepared by soaking 5 grams of dried trunks in 100 mL of hexane for 72 h. The trunk extract was filtered with Whatman filter

paper no. 1. The process was repeated for 3 times. Removal of the solvent were then done by evaporation. Stock solution (100 mg/mL) of the extract was prepared in DMSO, and stored at -20 °C until use.

### **Isolation of PBMCs**

This study was approved by The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University. PBMCs were obtained from 10 healthy volunteers, who were students at Faculty of Allied Health Sciences, Chulalongkorn University, Thailand. Each EDTA blood sample was diluted by the same volume of Hank's Balanced Salt Solution (HBSS). After that, the diluted blood sample was carefully layered on Lymphoprep (Axis-Shield, Oslo, Norway). The mixture was centrifuged at 800 x g for 20 min at room temperature. After centrifugation, the mononuclear cells formed a band then transferred it out. The cells were washed twice in PBS and resuspended in RPMI-1640 media (Hyclone, Logan, UT) with 100 U/mL of penicillin, 100µg/mL of streptomycin (Hyclone) and 10% fetal bovine serum (FBS). Determination of cell number and cell viability was performed using tryphan blue exclusion method [10].

### **Cell viability assay**

The effect of the extract on cell viability of PBMCs was determined by MTT assay, which was the measurement of enzyme activity that reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT, Bio Basic Inc., East Markham, ON, Canada) to formazan. The cells were seeded in a 96-well plate ( $1 \times 10^5$  cells/well). The *Vernonia cinerea* L. trunk extracts (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 µg/mL), and PBMCs were incubated for 24 h at 37 °C in a humidified condition containing 5% CO<sub>2</sub> (the percentage of DMSO in the experiment did not exceed 0.1). After that, 20 µl of 5 mg/mL MTT was added into each well in the 96- well plate and incubated for 4 h at 37 °C in a humidified environment containing 5% CO<sub>2</sub>. Subsequently, the medium was removed from every well, and 200 µl of DMSO was added to dissolve the formazan crystal. The absorbance was measured at 550 nm. The percentage of cell viability was calculated using the following formula:

$$\% \text{ cell viability} = [(\text{absorbance of treatment group} - \text{blank}) / (\text{absorbance of control group} - \text{blank})] \times 100$$

### **Cytokine measurement by ELISA**

The hexane extract of *Vernonia cinerea* L. trunk was tested for its effect on the cytokine secretion (TNF- $\alpha$ , IL-6 and IL-10). Cells were seeded in a 24-well plate ( $2 \times 10^6$  cells/well). The extract (100, 50, 25 and 12.5 µg/mL) and PBMCs were pre-incubated for 1 h at 37 °C in a humidified environment containing 5% CO<sub>2</sub>. Then, 10 ng/mL of lipopolysaccharide (LPS, *Eschericia coli* 055:B5, Sigma-Aldrich (St. Louis, MO) was added, and the cells were subsequently incubated for 24 h. At the end of the incubation period, the supernatants were collected and kept at -20 °C. The cultured supernatants were measured for cytokine by an enzyme-linked immunosorbent assay (ELISA) kit (PeproTech, Rocky Hill, NJ), according to the manufacturer's protocols.

**Detection of NF- $\kappa$ B subunit p65 nuclear translocation by confocal microscopy**

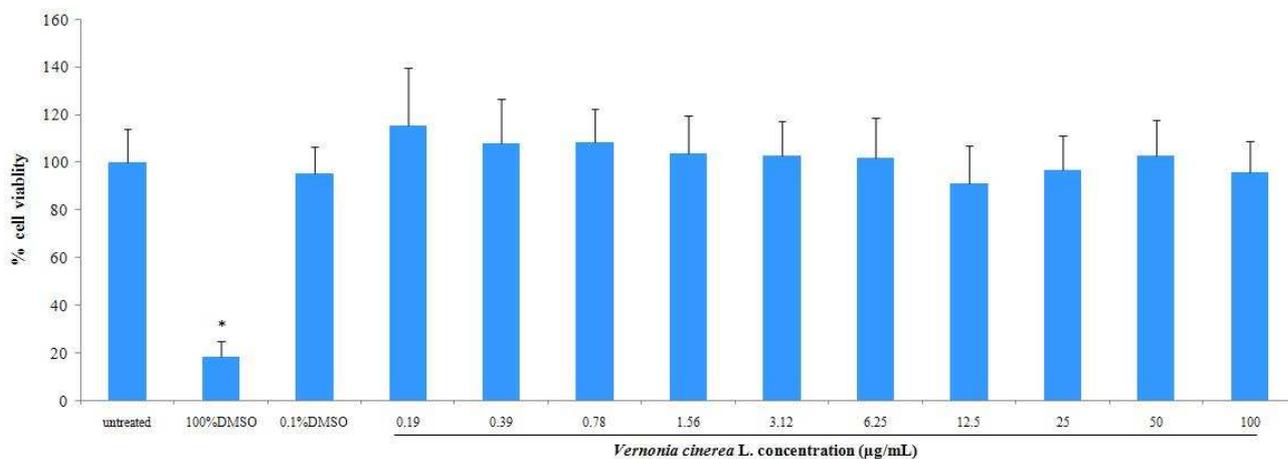
PBMCs were seeded in a 24-well plate ( $2 \times 10^6$  cells /well and pre-incubated with the hexane extract for 1 h. Subsequently, 10 ng/mL of LPS was added, and the cells were incubated for 24 h. After cells were fixed with freezing methanol for 5 min, they were washed three times with PBS, permeabilized and blocked with 5% BSA, 0.5% Triton X-100 in PBS for 1 h. After washing, cells were stained with 1: 500 dilution of rabbit anti-human p 65 antibody (Cell Signaling Technology, Danvers, MA) for 2 h at room temperature, washed to remove excess primary antibody, stained with 1:500 dilution of Alexa 555 – conjugated anti-rabbit IgG (Cell Signaling Technology) for 45 min, washed with PBS, stained with 1  $\mu$ g/mL of 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 1 h, and washed three times with PBS. Finally, each slide was covered with coverslip and observed using a confocal laser scanning microscope (LSM700, Carl Zeiss, Germany).

**Statistical analysis**

Data were analyzed using Student's *t*-test. Values of  $P < 0.05$  were considered as significant. The data represented as mean  $\pm$  S.E.M.

**RESULTS AND DISCUSSION****1. The hexane extract of *Vernonia cinerea* L. trunk possessed no effect on PBMCs viability.**

To determine the effect of crude extract on cell viability, PBMCs were incubated with different concentrations of the hexane extract of *Vernonia cinerea* L. trunk (from 0 to 100  $\mu$ g/mL) for 24 h. Using MTT assay, no effect of the extract was observed on the cell viability at the concentration range of 0.19 to 100  $\mu$ g/mL. The lowest cell viability of human PBMCs was  $95.77 \pm 12.91\%$  as shown in Figure 1.



**Figure 1** Effect of the hexane extract of *Vernonia cinerea* L. trunk on cell viability. PBMCs were treated with different concentrations of extract (0 to 100  $\mu$ g/mL) for 24 h, and viability was determined by MTT assay. 0.1% DMSO was used as a control solvent. Data are shown as means  $\pm$  SEM of triple determinations. (\*  $P < 0.05$  compared with untreated cell)

## 2. The hexane extract of *Vernonia cinerea* L. trunk significantly reduced pro-inflammatory cytokine level, IL-6, but not TNF- $\alpha$ .

Based on the cytokine measurement by ELISA, we found that hexane extract of *Vernonia cinerea* L. trunk at 12.5  $\mu\text{g/mL}$  significantly reduced IL-6 level ( $P < 0.05$ ) as shown in Figure 2. Specifically, about  $57.89 \pm 2.54\%$  reduction was observed. However, there was no change in TNF- $\alpha$  level under the same treatment conditions as shown in Figure 3.

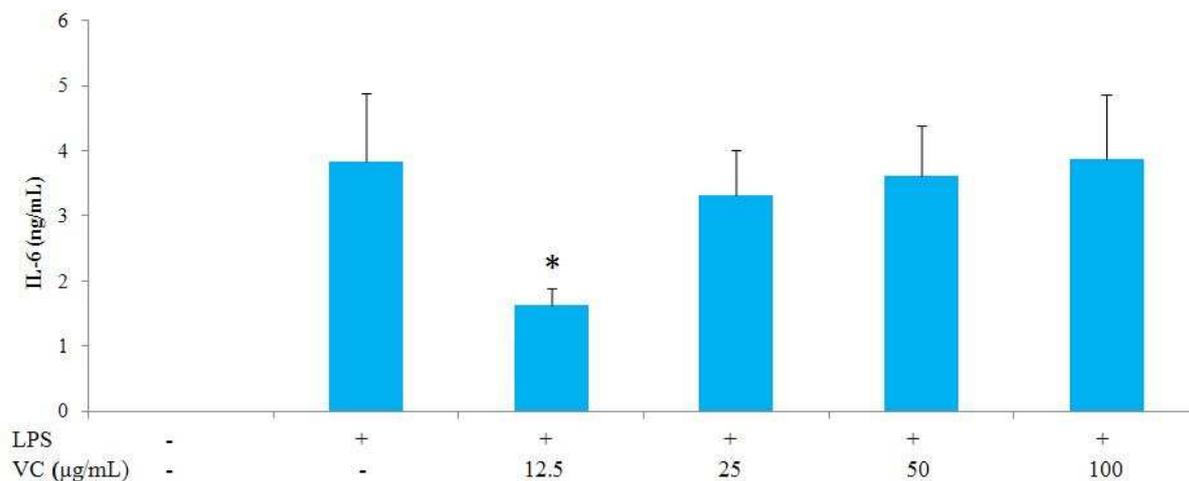


Figure 2 The effect of hexane extract of *Vernonia cinerea* L. trunk on IL-6 secretion in LPS-stimulated PBMCs control compared with treated cells by various concentrations. Measurement using ELISA was performed in cultured supernatants. Data are shown as means  $\pm$  SEM of triple determinations. (\*  $P < 0.05$  compared with LPS)

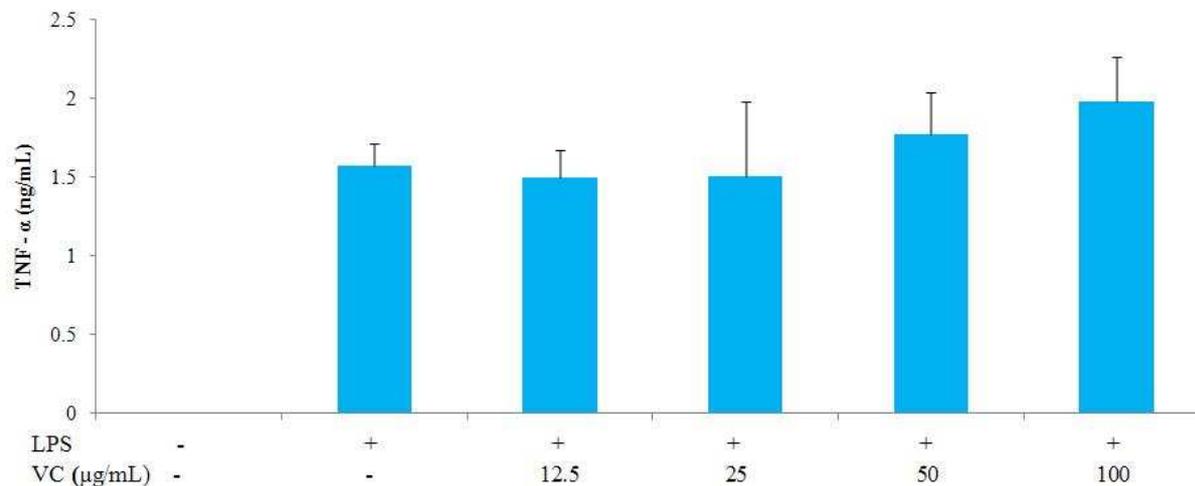
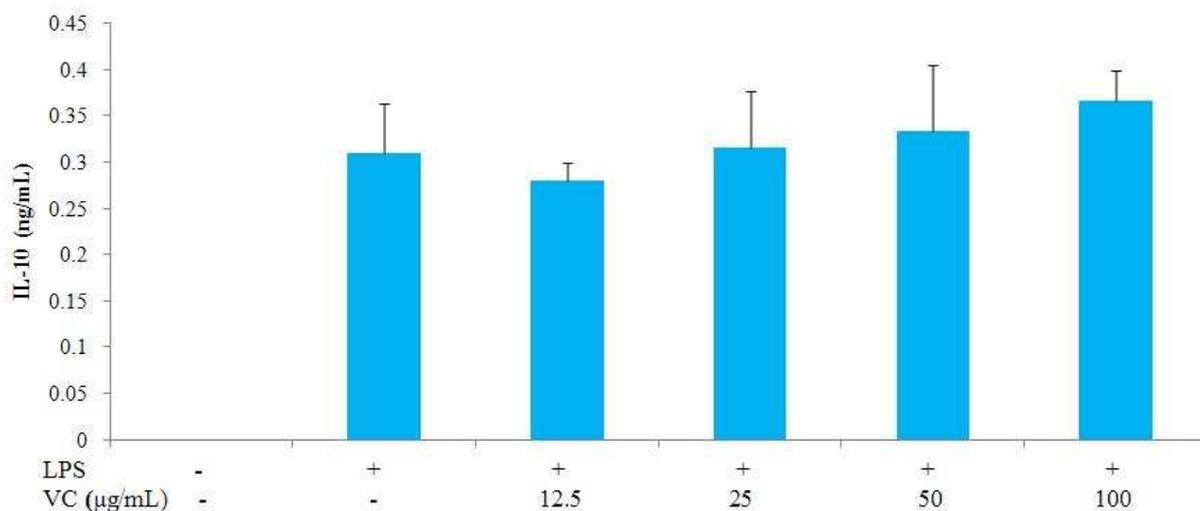


Figure 3 The effect of hexane extract of *Vernonia cinerea* L. trunk on TNF- $\alpha$  secretion in LPS-stimulated PBMCs control compared with treated cells by various concentrations. Measurement using ELISA was performed in cultured supernatants. Data are shown as means  $\pm$  SEM of triple determinations.

### 3. The hexane extract of *Vernonia cinerea* L. trunk possessed no effect on anti-inflammatory cytokine level, IL-10.

According to the cytokine secretion measurement, we found that the hexane extract did not alter the IL-10 level as compared to the control (Figure 4).



**Figure 4** The effect of hexane extract of *Vernonia cinerea* L. trunk on IL-10 secretion in LPS-stimulated PBMCs control compared with treated cells by various concentrations. Measurement using ELISA was performed in cultured supernatants. Data are shown as means  $\pm$  SEM of triple determinations.

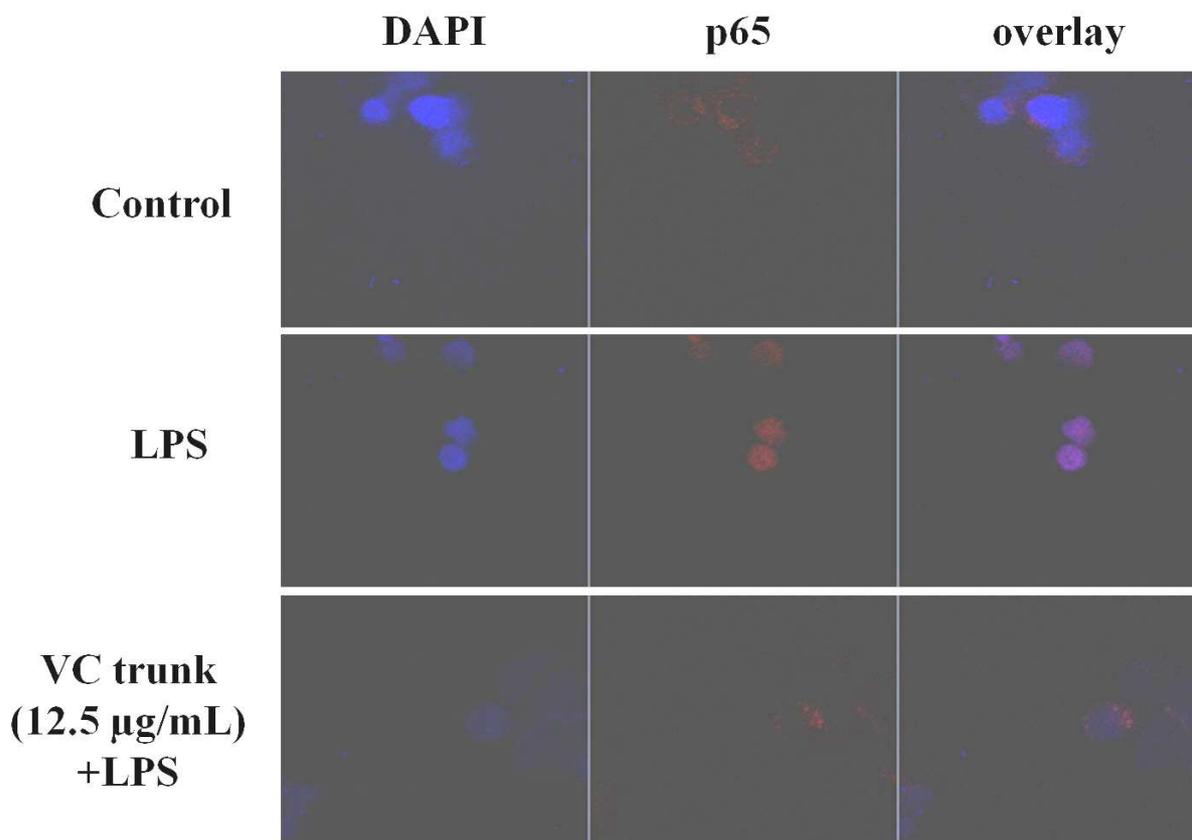
### 4. NF- $\kappa$ B nuclear translocation was inhibited by the hexane extract of *Vernonia cinerea* L. trunk.

To investigate the effect of the hexane extract of *Vernonia cinerea* L. trunk on activation of the nuclear translocation of NF- $\kappa$ B, which has been known to induce both of IL-6 and TNF- $\alpha$  secretions, we used immunocytochemistry analysis in combination with confocal laser scanning microscopy. LPS stimulated the translocation of NF- $\kappa$ B subunit p65 from the cytoplasm to the nucleus of PBMCs (Figure 5). Interestingly, the extract inhibited NF- $\kappa$ B subunit p65 translocation in LPS-stimulated PBMCs. Based on this finding, reduction of IL-6 secretion might be due to the inactivation of NF- $\kappa$ B signaling via inhibition of nuclear translocation.

In the present study, evaluation of the toxicity of hexane extract of *Vernonia cinerea* L. trunk on human PBMCs found that the hexane extract showed no influence on cell viability. The highest concentration of hexane extract used, 100  $\mu$ g/mL, exerted no effect on cell death. In accordance with another investigation, the methanolic extract of *Vernonia cinerea* L. trunk at 100 mg/kg resulted in a mild toxicity in animal model [11].

Evaluation of immunomodulatory effect of *Vernonia cinerea* L. trunk on LPS-stimulated PBMCs displayed that the hexane extract at the concentration of 12.5  $\mu$ g/mL significantly decreased LPS-induced IL-6 production ( $P < 0.05$ ),  $57.89 \pm 2.54\%$ . On the contrary, no effect on LPS-induced TNF- $\alpha$  production was found under the same concentration tested. The reduction

of IL-6 level might be resulted from anti-inflammatory activity of stigmasterol, the compound in the whole plant of *Vernonia cinerea* L. [12].



**Figure 5** The images from confocal laser scanning microscope showed that the hexane extract inhibited the NF- $\kappa$ B nuclear translocation in LPS-stimulated PBMCs. PBMCs were pre-treated with the extract for 1 h and stimulated with LPS for 24 h. The NF- $\kappa$ B localization was determined using immunocytochemistry and confocal laser scanning microscope. After fixation, cells were stained with anti-p65 antibody, followed by Alexa-555 conjugated antibody (red). Nucleus was visualized with DAPI (blue). Control was the group of untreated cells.

We found that the hexane extract of *Vernonia cinerea* L. trunk at a low concentration displayed an anti-inflammatory effect by inhibiting LPS-induced IL-6 secretion in human PBMCs via downregulation of NF- $\kappa$ B nuclear translocation. LPS can induce the inflammation via NF- $\kappa$ B, a transcription factor that contributes to the transcription of TNF- $\alpha$  and IL-6 genes, thus leading to the secretion of TNF- $\alpha$  and IL-6 [3]. Although inhibition of NF- $\kappa$ B activation was experimentally evident, reduction of pro-inflammatory cytokine production was found only for IL-6, not TNF- $\alpha$  level. This finding could be possible since the expression of IL-6 and TNF- $\alpha$  was regulated by not only NF- $\kappa$ B, but also other transcription factors. For instance, LPS-induced TNF- $\alpha$  factor (hLITAF) was shown to mediate TNF- $\alpha$  transcription, thus leading to TNF- $\alpha$  secretion [13]. Nevertheless, higher concentrations of the same extract (25, 50 and 100  $\mu$ g/mL) did not alter the secretion of IL-6. Possibly, the hexane extract of *Vernonia Cinerea* L. trunk at

high concentrations may contain higher levels of certain constituents, thus exerting different effects. In other words, this phenomenon may be occurred because of a masking effect.

In conclusion, the hexane extract of *Vernonia Cinerea* L. trunk at the concentration of 12.5 µg/mL significantly decreased the IL-6 level, and this particular reduction might be regulated via an inhibition of NF-κB nuclear translocation.

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