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Research Article

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Crinum latifolium extract inhibits lipopolysaccharide-induced inflammation in human macrophages

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Abstract

***Introduction**: Crinum latifolium is a natural plant showing the anti-inflammatory effects. We aimed to evaluate the effects of Crinum latifolium extract on proinflammatory cytokine production and maturation of early and late endosomes in the lipopolysaccharide (LPS)-induced human macrophages at light microscopic and biochemical levels. **Material and Methods**: The effects of Crinum latifolium extract in human macrophages stimulated with LPS was evaluated to optic densities of the lysosome-associated membrane protein-1 (LAMP-1) and early endosome antigen 1 (EEA1) by immunohistochemistry staining and to level of the proinflammatory cytokine interleukin (IL)-6 by enzyme-linked immunosorbent assay (ELISA). **Results**: Crinum latifolium group exhibited a significant decreased in the levels of IL-6 in the supernatant as compared to the LPS group for 2 and 72 hours ($p<0.0001$). Crinum latifolium group exhibited a significant increased in the optic density of the EEA1 and LAMP-1 in coverslips as compared to the control and lipopolysaccharide groups after 2 and 72 hours ($p<0.0001$). **Conclusions**: Crinum latifolium may a therapeutic plant in the inflammatory diseases, such as sepsis, through anti-inflammatory effects, such as decrease in production of the proinflammatory cytokine and increase in maturations of the early and late endosomes in macrophages.*

***Keywords**: inflammation, macrophage, interleukin-6, Crinum latifolium extract, endosomes*

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Introduction

Amaryllidaceae species are wild and cultivated plants in different regions around the world (1). Amaryllidaceae species are essentially due to the alkaloids and flavonoids content. *Crinum species* are genus of the large *Amaryllidaceae family and* used in folk medicine (1, 2). The alkaloids isolated from *Crinum species* show significant antimicrobial activity against the Gram+ bacteria and Gram– bacteria (1). All the alkaloids also show antioxidant activity and anti-inflammatory potential (1, 3, 4).

Severe inflammation caused by chronic respira-

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tory diseases, cancer, obesity (5), stroke, heart disorders, and diabetes diseases (6, 7) lead to death of the patients. Therefore, controlling inflammation during chronic diseases remains therapeutically significant. Current anti-inflammatory therapies include steroids and non-steroidal anti-inflammatory drugs (8, 9). However, these drugs are associated with various side effects, which may lead to the risk of morbidity and even mortality (10, 11).

Macrophages form the foremost line of the immune system to protect from the infections of viruses and bacteria (12). The activated macrophages play a significant role in immune response, and abnormal macrophage activation frequently leads to immune and inflammatory diseases. However, the mechanisms of macrophage activation are not completely understandable (13, 14).

The LPS induced macrophage activation plays an important role in many chronic inflammations. In the inflammatory response, macrophages play an important role by stimulating other cells of the immune system to produce the proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-6. The bacterial endotoxins and many other factors elaborated in various infectious diseases are shown to induce the expression of inflammation-related cytokines in the macrophages (15, 16). The macrophages may appear as potential targets for the treatment of diseases (17). However, dysregulated macrophage activation can also result in inflammatory diseases (18). The targeted macrophage activation would also be an efficient approach for the therapy of inflammatory diseases (19).

Endosomes are sub-cellular organelles, and are associated with elimination of pathogenic organisms during the *inflammatory* response. The macrophages utilize from immune mechanisms to eliminate many intracellular bacterial pathogens. (20, 21). The maturation of phagosome in macrophages depends on the endosomal pathway. The newly formed phagosome, which is incapable of killing, is remodeled by fusion with early and late endosomes, and finally lysosomes (Figure 1) (22). The phagosome maturation may affect the phagosomal escape of the intracellular bacterial pathogens. The slow speed phagosome maturation will give more time for intracellular bacterial pathogens to escape (23).

The aim of this study was to investigate the effects of *Crinum latifolium* extract on production of proinflammatory cytokine and maturation of early and late endosomes in the LPS-induced human macrophages.

Materials And Methods

Cell culture and treatment

The human macrophage primary cell line derived from the human peripheral blood (36070-01, frozen vial, Celprogen Inc. San Pedro CA 9073) was cultured and subcultured at 37°C in 5% CO2 in the complete growth medium with serum (M36070-01S, Celprogen Inc. San Pedro

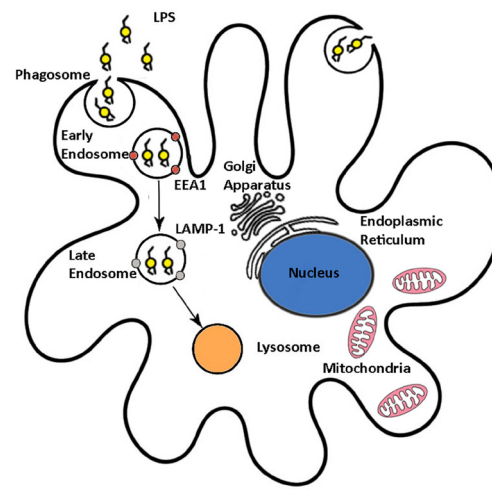


Fig. 1. Schematic illustration of endosome trafficking in LPS-stimulated macrophage. EEA1 is localized on the membrane of early endosome. LAMP-1 is localized on the membrane of late endosome.

CA 9073) and antibiotic-antimycotic (15240062, Gibco, Thermo Fisher Scientific, USA). The human macrophage cells become a near 80% confluent, and it was counted at a Neubauer chamber with the trypan blue solution. The human macrophage cells were seeded at approximate density of 20000 cells per well of six-well plates (38016, Falcon, STEMCELL Technologies, Inc., CA). The experiments (coverslips and their supernatants) were repeated three times for all groups.

The human macrophages with unstimulated (control), or activated by the LPS (Escherichia coli, 055 : B5; Sigma) were cultured in the absence or presence of the *Crinum latifolium* extract (25 mg/ml) after 2 and 72 hours. After incubation, the optic density of the EEA1 and LAMP-1 in the human macrophages was analyzed using the immunohistochemistry staining of coverslips in the plates. In addition, the IL-6 levels in the supernatants were measured with ELISA.

Preparation of the Crinum latifolium extract

The dry form of *Crinum latifolium* leaves from the CriLa® capsules (250 mg) was used provided by the Thien Duoc Company Ltd. (Ho Chi Minh City, Vietnam) (24). In this study, dry form of a CriLa® capsule was extracted with cell culture media and sterile filter.

Immunohistochemical staining

The coverslips were rinsed with phosphate buffered saline (PBS, 003002, Thermo Fisher Scientific, USA), fixed with 4% paraformaldehyde (25) solution, rinsed and stored in PBS at 4°C until the EEA1 and LAMP1 immunohistochemistry staining.

The coverslips were immunostained by rabbit anti-human EEA1 polyclonal (1 : 100 dilution, H-300, sc-33585, Santa Cruz, CA) and the mouse anti-human LAMP1 monoclonal antibodies (1:100 dilution, E-5, sc-17768, Santa Cruz, CA) primary antibodies. The coverslips were permeable in PBS containing the Triton X-100, blocked with BSA at room temperature, and incubated with primary antibodies at 4°C overnight. The coverslips were incubated with a biotinylated anti-mouse or anti-rabbit IgG (sc-2017 and sc-2018, Santa Cruz, CA), then avidin biotinylated or streptavidin with horseradish peroxidase complex in normal goat serum. The coverslips were then stained with chromogen 3-amino-4-ethylcarbazole (AEC). The coverslips were counterstained with or without hematoxylin and mounted. Finally, immunostained coverslips were imaged on the microscope for quantitative analysis.

IL-6 ELISA

The analysis of IL-6 was performed in the culture supernatants. The culture supernatants were centrifuged. In the supernatants, IL-6 level was assayed using appropriate ELISA kit (KHC0061, Invitrogen, USA). The analyses were performed according to the instructions and read by the absorbance microplate reader (BioTek, EL x 800). The concentration of total protein in the supernatant was detected by the Breadford method.

Statistical analysis

The imaging was performed using an Olympus microscope (BX61) with the camera system (DP72). The randomly chosen digital images were analyzed with Aperio ImageScope image analysis software. The positive pixel count algorithm was determined from five views within the images. The values are given as the mean ± standard error. The groups were assessed using a one-way ANOVA followed by the Tukey post hoc test. The p-values < 0.05 were considered statistically significant. SPSS (Statistical Package for the Social Sciences, version 28.0, SPSS Inc., Chicago, IL, USA) software was used for *statistical* analysis of data.

Results

The macrophages of the control group remained rounded and had a smaller cell circumference than macrophages of the LPS and the *Crinum latifolium* groups. In the LPS and *Crinum latifolium* groups, the macrophages were enlarged, swollen and more protruding than macrophages of the control group (Figure 2, A-F).

The optic density of EEA1 was significantly increased for the *Crinum latifolium* group when compared to the control group after 2 and 72 hours of LPS stimulation ($p<0.0001$, Figure 2, A, B, E, F and Figure 4, A). In the *Crinum latifolium* group, the optic density of EEA1 was a significantly increased when compared to the LPS group after 2 and 72 hours of LPS stimulation ($p<0.0001$, Figure 4, A). In the LPS group, the optic density of EEA1 was significantly increased after 2 hours of LPS stimulation ($p<0.0001$, Figure 2, C), but not significantly when compared to the control group after 72 hours of LPS stimulation ($p=0,121$, Figure 2, D).

The optic density of LAMP1 was significantly increased in the *Crinum latifolium* group when compared to the control group after 2 and 72 hours of LPS stimulation ($p<0.0001$, Figure 3, A, B, E, F and Figure 4, B). In the *Crinum latifolium* group, the optic density of LAMP1 was significantly increased when compared to the LPS group after 2 and 72 hours of LPS stimulation ($p<0.0001$, Figure 4, B). There was significant increase in the optic density of LAMP1 in the LPS group than the control group after 2 hours of LPS stimulation ($p<0.0001$, Figure 3, C) but not significant when compared to the control group after 72 hours of LPS stimulation ($p= 0.2405$, Figure 3, D).

In the LPS group, the level of IL-6 was significantly increased when compared to the control and the *Crinum latifolium* groups after 2 and 72 hours of LPS stimulation ($p<0.0001$, Figure 4, C). There was **no** significant difference between with the *Crinum latifolium* and control

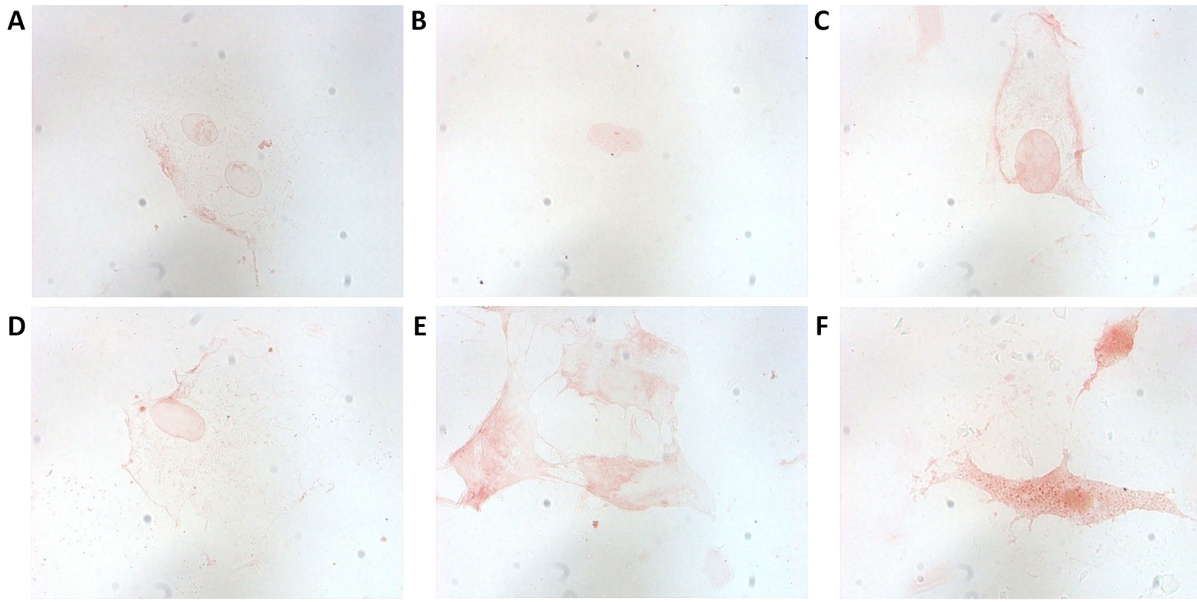


Fig.2. Representative images of the EEA1 immunohistostaining in each group after 2 and 72 hours of LPS stimulation (A-F). In the control (A and B), LPS (C and D) and *Crinum latifolium* (E and F) groups shown EEA1-positive (red signal) staining of macrophages.

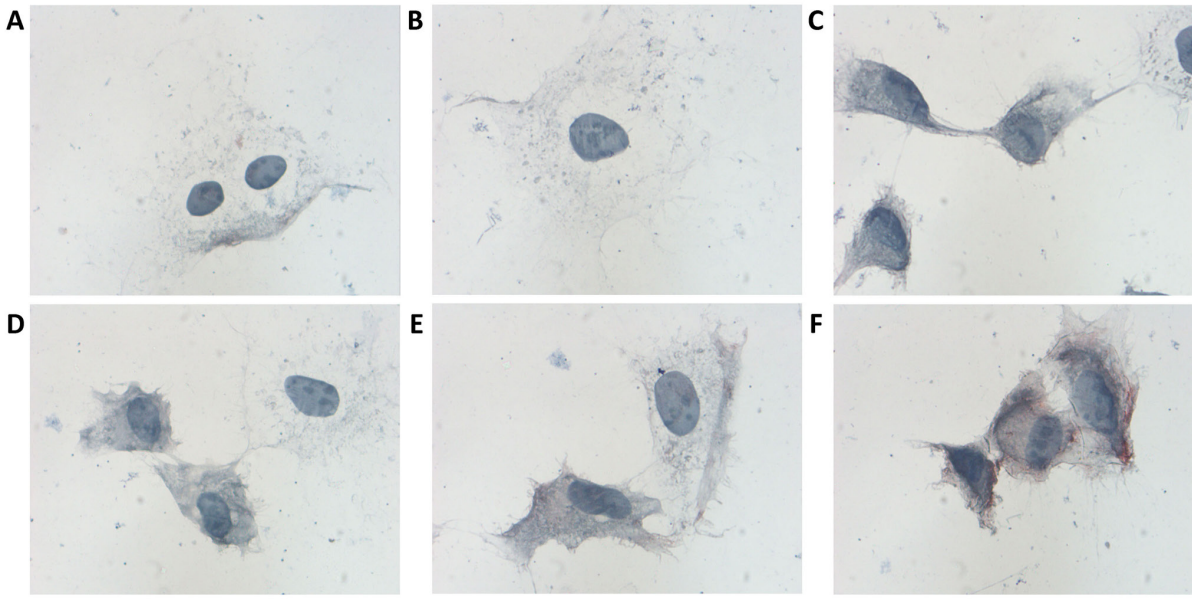
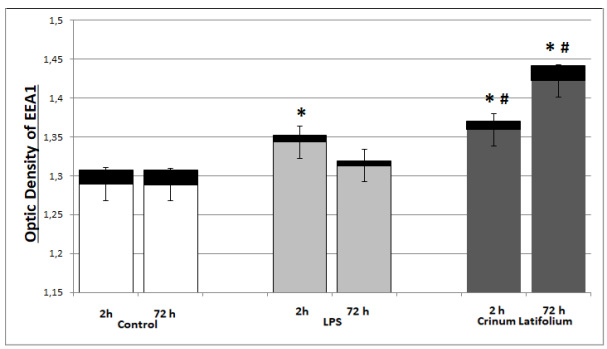
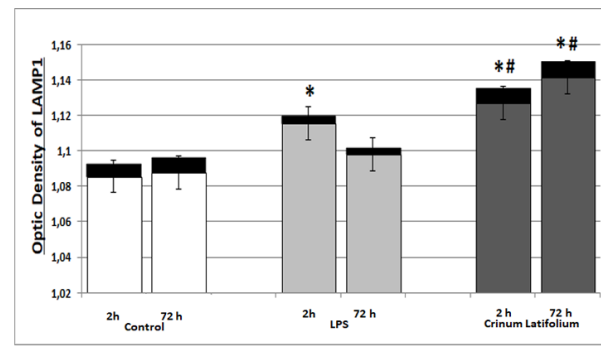


Fig.3. Representative images of the LAMP1 immunohistostaining in each group after 2 and 72 hours of LPS stimulation (A-F). The LAMP1 immunoreactivity (red signal) and blue hematoxylin counter staining seen to the macrophages in the control (A and B), LPS (C and D) and *Crinum latifolium* (E and F) groups.





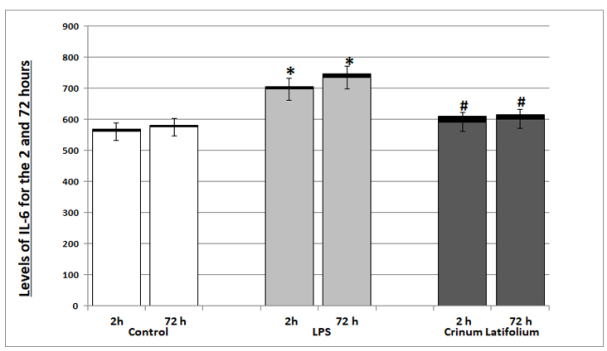


Fig.4. The figures represent the mean (bars) + SEM (error bars) in each group for the EEA1 (A) and LAMP1 (B) optic density after 2 and 72 hours of LPS stimulation. (C) The analysis of the IL-6 levels in the control, LPS and *Crinum latifolium* groups using ELISA after 2 and 72 hours of LPS stimulation. * and # indicates a significant difference compared to the control and LPS groups, respectively (p< 0.0001).

groups after 2 and 72 hours of LPS stimulation ($p=0.0038$ and $p=0.0149$, respectively, Figure 4, C). However, the IL-6 of the *Crinum latifolium* group was significantly decreased in comparison to the LPS group after 2 and 72 hours of LPS stimulation ($p<0.0001$, Figure 4, C).

Discussion

Macrophages are phagocytic cells that are involved in the protection against bacteria and viruses, antitumour immunity, inflammatory signaling, and wound healing (26). Macrophages are responsible for the release of proinflammatory cytokines such as IL-1, TNF-α, IL-6. Macrophages play an important role in immune response, and abnormal activation of macrophages frequently leads to inflammatory and immune diseases (13, 14). *Crinum latifolium* belongs to *Amaryllidacea* and is generally used in *traditional medicine* in different regions around the world. Studies have produced some evidence to explain the immunostimulation effects of *Crinum latifolium* extract (1, 27). However, the mechanisms of the action of *Crinum latifolium* extract are unclear. In the current study, there was a significant increase in the IL-6 level of the LPS group compared to the control group after 2 and 72 hours of LPS stimulation. In contrast, the IL-6 level of *Crinum latifolium* extract was slightly higher than the *control group*, *but* it was *not significant* after 2 and 72 hours of LPS stimulation.

Early endosomes are specialized in cellular compartments that take and sort *endocytic* material to late endosomes and lysosomes (28). The existence of EEA1 on phagosomes is essential for obtaining the late endocytic characteristics (29). The EEA1 contributes to the maturation of phagosome to lysosomes (30). All cells expressed markers of the early and late endosomes at basal levels (31). The EEA1 protein is present in early endosome on the first hours of infection. LPS promotes decrease density of EEA1 after 24 hours (32). We found high expression of LAMP1 in the LPS group after 2 hours of LPS stimulation, while in LAMP1 there was a decrease after 72 hours of LPS stimulation. Our results also indicate that the EEA1 optic density was significantly increased in the Crinum Latifolium group when compared to both the control and LPS groups after 2 and 72 hours of LPS stimulation. In addition, the LAMP1 optic density was increased in the *Crinum latifolium* group when compared to the control and LPS groups after 2 and 72 hours of LPS stimulation. In the LPS group, the levels of EEA1 and LAMP1 were increased after 2 hours of LPS stimulation, but not significantly in comparison to the control group after 72 hours of LPS stimulation. These results suggest that maturation of endosomes decreased in the LPS group after 72 hours of LPS stimulation.

Conclusions

This study has identified that *Crinum latifolium* decreases proinflammatory cytokine IL-6 expression and increases early and late endosomes possibly through activating autophagy in the LPS-induced human macrophages. Therefore, *Crinum latifolium* could play an important anti-inflammatory role on the macrophages. The findings have shown that *Crinum latifolium* is involved in the situations during immune responses, which include the production of proinflammatory cytokine IL-6 and expressions of EEA1 and LAMP1 proteins in the endosomes. Thus, *Crinum latifolium* could be a possible therapeutic plant to regulate the production of proinflammatory cytokine and maturations of early and late endosomes in inflammatory diseases. Further studies are required to determine whether the efficacy of *Crinum latifolium* could have effects on the other proinflammatory cytokines production and endosomes of the macrophages in the different inflammatory diseases models.

Abbreviations

EEA1 – early endosome antigen 1
LAMP-1 – lysosome-associated membrane protein-1
IL – interleukin
LPS – lipopolysaccharide
ELISA – enzyme linked immunosorbent assay
TNF-α – tumor necrosis factor alpha
AEC – 3-amino-4-ethylcarbazole

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Authors' contribution

SK – Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing–original draft preparation, Writing-review and editing Visualization, Supervision, Project administration, Funding acquisition
YC – Methodology, Validation, Formal analysis, Writing-review and editing, Funding acquisition
SC – Methodology, Validation, Formal analysis, Writing-review and editing, Funding acquisition
All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Conflict of interest

Conflict of interests The authors declare that they have no conflict of interests.

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