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Stimulatory Activity of *Nelumbo Nucifera* Gaertn. Water Extract on Macrophage J774A.1 Cells

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Abstract

Pollen from Lotus (*Nelumbo nucifera* Geartn.) is wildly used in traditional medicine for increase body energy, relieve tiredness and promote function of heart. This study aimed to investigate stimulatory activity of the water extract of *Nelumbo nucifera* Gaertn. on macrophage functions.

Mouse macrophage J774A.1 cells were treated with the water extract for 4-24 h. Nitric oxide (NO) from the supernatant of the treated cells was determined by Griess reaction assay. Expression of inducible nitric oxide synthase (iNOS) and cytokines was determined by RT-PCR.

The results showed that the extract at 1.56 – 100 μg/ml significantly increased production of nitric oxide which plays roles in phagocytosis for destroying microbes and in acute inflammation without any effect on cell viability. Effect of the extract on NO production correlated to its effect on induction of iNOS which is the enzyme responsible for NO production in activated macrophages. The extract also increased expression of cytokines which are markers of macrophage activation. These cytokines were interleukin-1β (IL-1β), Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). The results from this study reveal that the water extract of lotus pollen has immunostimulatory effect on macrophage cells.

Keywords: *Nelumbo Nucifera* Gaerth., Macrophage, Nitric oxide, Cytokines

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Introduction

The immune system consists of a network of cells, tissues and organs that work together to maintain body's homeostasis and protect the body from microorganisms or foreign particles. There are two main types of immune response; innate and adaptive immunity. Innate immunity is the first line of defense against pathogens. It immediately reacts to the pathogens with low specificity and in generic ways. Adaptive immunity is specific immune response with memory and provides potent activity for eliminating the pathogens. Both types of immunities always cooperatively work in complex interactions. Cells in innate immunity initiate adaptive immunity by acting as angiten presenting cells to lymphocytes. Lymphocytes in adaptive immunity always need cells in innate immunity for their effector functions to eliminate pathogens.

Macrophages are immune cells which function in both innate and adaptive immunity. They are main tissue phagocytes responsible for eliminating invading pathogens. They recognize pathogens by their pattern recognition receptor (PRRs) and become activated macrophages. Phagocytosis is an important function of macrophages and activated macrophages for destroying pathogens. After recognizing pathogens through PRRs, macrophages engulf the pathogens, form phagosomes containing pathogens in the cells, fuse the phagosomes with lysosomes to become phagolysosomes, induce expression and production of enzymes responsible for reactive oxygen species (ROS) and reactive nitrogen species (RNS), kill engulfed pathogens in phagolysosomes by oxygen-dependent pathway using ROS and RNS and by oxygen-independent pathway by using lysozymes in lysosomes. Activated macrophages express and produce numerous mediators with various functions in both innate and adative immunity. Some of these mediators are often used as the markers for macrophage activation. These include cytokines TNF-α, IL-1, IL-6, chemokines IL-8, MCP-1 and MIP-1α, and NO. Macrophages can present destroyed pathogen in the form of antigenic peptides to specifically activate T lymphocytes. This initiates adaptive immunity against the pathogens. Activated T lymphocytes play role in adaptive immunity by secreting several cytokines to activate various immune cells as well as macrophages to cooperatively eliminating the pathogens.

Lotus (*Nelumbo nucifera* Geartn.) is an aquatic plant wildely distributed in Asia and India. Many parts of this plant are used as food and medicines. Lotus pollen has been used in Thai traditional medicine for increasing body energy, relieving tiredness and promoting heart function. It is prepared for use by boiling with water, as crude power in pills, and in combination with other herbal medicines as recipe. No evidence of pharmacological study on lotus pollen.

Objective

This study intended to investigate pharmacological effect of the water extract of *N. nucifera* pollen on macrophages.

Materials and Methods

Materials

Plant extract

The water extract of *N. nucifera* was prepared by heat in distilled water at the ratio of 1:5 (*N. nucifera* powder : water) at 80˚C for 15 minutes. The supernatant was filtered and dried by lyophilization. The water extract powder was stored at -20˚C until used. In all experiments, the extract was dissolved and diluted to various concentrations in incomplete Dulbeco's Modified Eagle's Medium (DMEM).

Cells

The murine macrophage J774A.1 cells were obtained from American Type Culture Collection (ATCC). The cells were maintained at 37 ^{o}C in DMEM containing 10% fetal bovine serum (FBS), 100 units/ml penicilin and 100 units/ml streptomycin.

Methods

In all experiments, J774A.1 cells were used at 4 x 10^5 cell/ml. DMEM and 100 ng/ml lipopolysaccharide (LPS) were used as the negative and the positive controls, respectively.

Determination of NO production and Cell viability

J774A.1 cells were treated with 1.56 – 100 µg/ml water extract for 24 h. The supernatants of the treated cells were collected for determining NO production in nitrite form according to the protocol in Griess reaction assay kit (Promega, USA). The concentrations of NO were calculated from a standard curve prepared from standard sodium nitrite solutions. The remaining treated cells were tested for cell viability by resazurin reduction assay.

Determination of iNOS, IL-1β, IL-6 and TNF-α mRNA expression by RT-PCR

J774A.1 cells were treated with 12.5 – 100 µg/ml water extract for 4 or 24 h. The treated cells were collected for isolating total RNA by TRIzol reagent and converting to complementary DNA (cDNA) by ImProm-II™ reverse transcription system kit (Promega, USA). The cDNA samples were used to amplify PCR products of iNOS, IL-1β, IL-6 and TNF-α with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel electrophoresis and measured their densities by gel documentation.

Statistical analysis.

All data were presented as means ± standard error of means (S.E.M.) from at least three independent experiments. Statistical significance of the data was analyzed by One-way ANOVA with Least Significant Difference (LSD) post hoc test. The p-value less than 0.05 were regarded as statistically significant.

Result

Effect of the water extract of *N. nucifera* on NO production

Activated macrophages generated NO to function as toxic mediator to pathogen during phagocytosis and as a mediator involves in inflammatory process. NO is usually not produced at resting state as presented as media treated condition in Fig. 1. It is one of markers of activated macrophages. The water extract at 12.5 – 100 µg/ml significantly increased NO production in macrophage J774A.1 cells in a concentration dependent manner when compared with untreated control (Fig. 1).

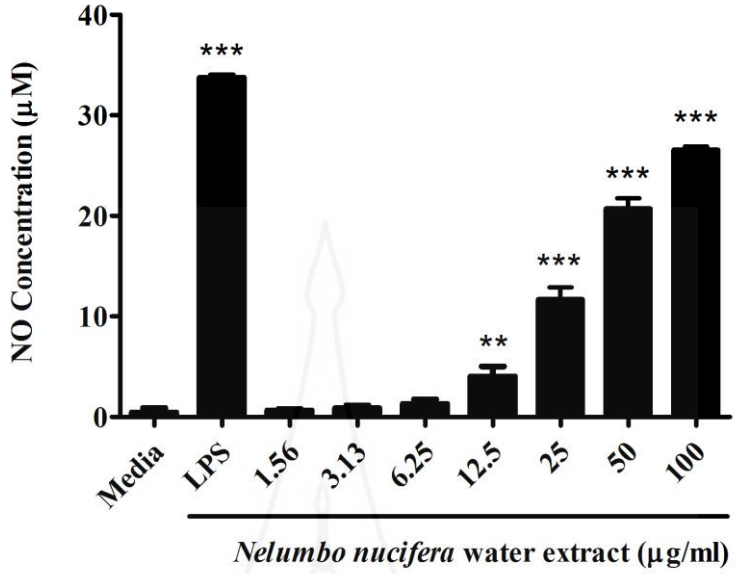

Figure 1. Effect of the water extract of *N. nucifera* at 1.56 – 100 µg/ml on NO production in macrophage J774A.1 cells. The data present as mean ± S.E.M from three independent experiments (n=3). **p<0.01, ***p<0.001 compared to untreated control.

Effect of the water extract of *N. nucifera* on cell viability

The stimulatory effect of the extract on NO production did not come from its proliferative effect. Viability of the extract treated cells was similar to the untreated control as presented in Fig. 2.

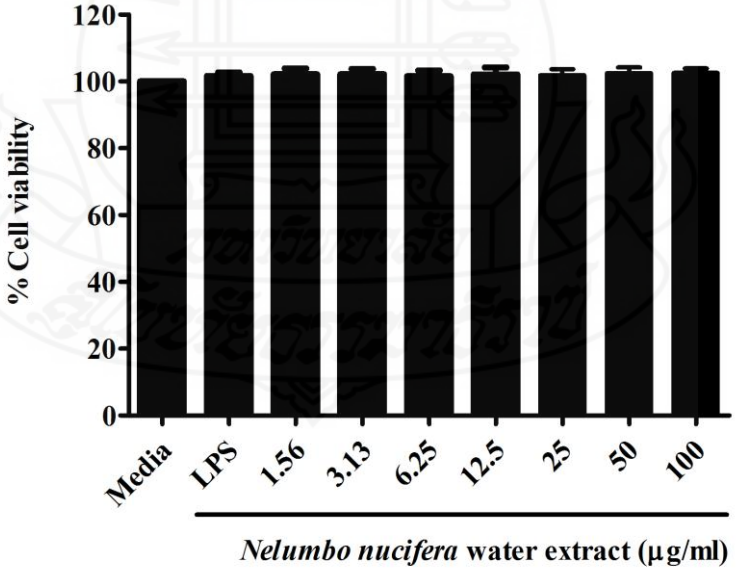

Figure 2. Effect of the water extract of *N. nucifera* at 1.56 – 100 µg/ml on cell viability of macrophage J774A.1 cells. The data present as mean ± S.E.M from three independent experiments (n=3).

Effect of the water extract of *N. nucifera* on mRNA expression of iNOS

The stimulatory effect of the water extract on NO production was confirmed by evaluating iNOS expression. The water extract at 12.5 – 100 µg/ml significantly increased mRNA expression of iNOS as presented in Fig. 3.

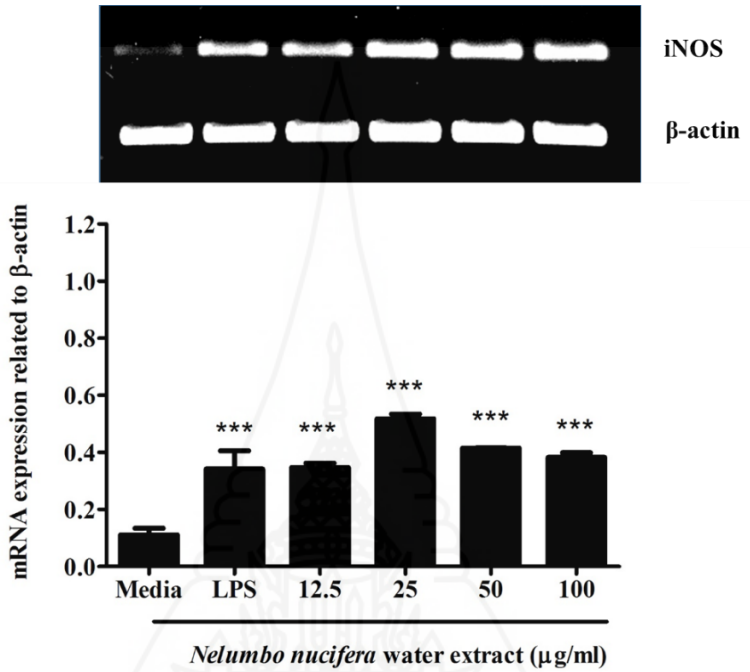

Figure 3. Effect of the water extract of *N. nucifera* at 12.5 – 100 µg/ml on mRNA expression of iNOS in J774A.1 after 24 h of treatment. The data present as mean ± S.E.M from three independent experiments (n=3). **p<0.001 compared with untreated control.

Effect of the water extract of *N. nucifera* on mRNA expression of IL-1β, IL-6, and TNF-α

The water extract at 12.5 – 100 µg/ml significantly stimulated mRNA expression of IL-1β (Fig. 4) and TNF-α (Fig. 5). At 50 and 100 µg/ml, the extract significantly stimulated mRNA expression of IL-6 (Fig. 6).

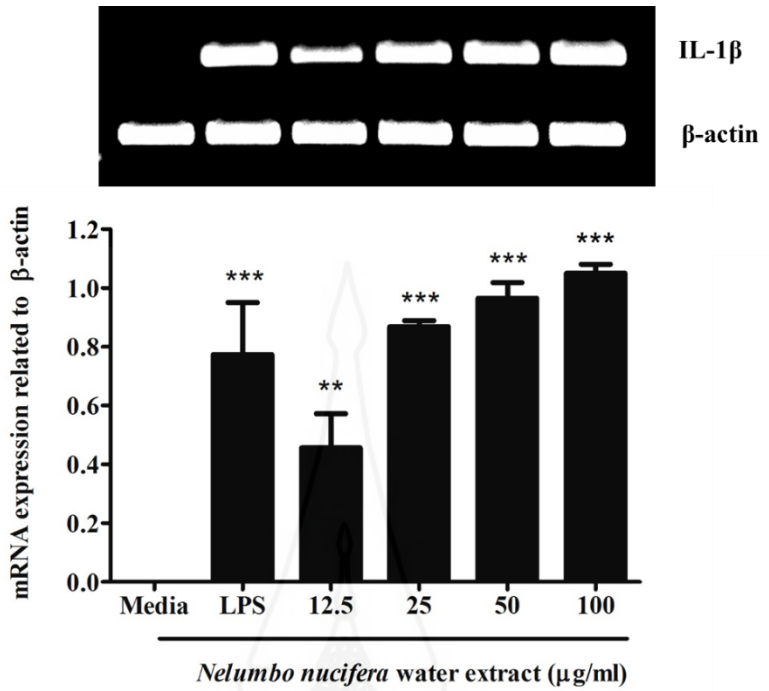

Figure 4. Effect of the water extract of *N. nucifera* at 12.5 – 100 µg/ml on mRNA expression of IL-1β in J774A.1 after 4 h of treatment. The data present as mean ± S.E.M from three independent experiments (n=3). **p<0.01, ***p<0.001 compared with untreated control.

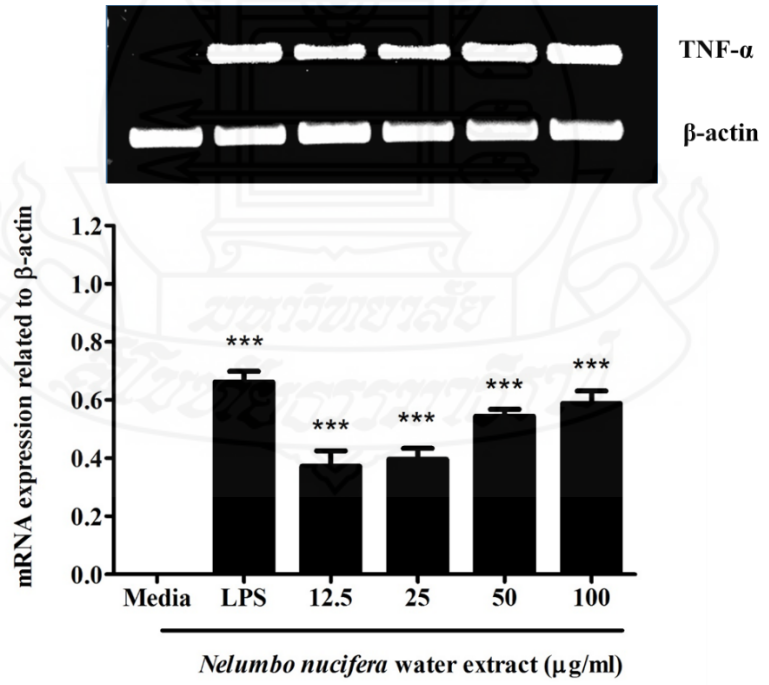

Figure 5. Effect of the water extract of *N. nucifera* at 12.5 – 100 µg/ml on mRNA expression of TNF-α in J774A.1 after 4 h of treatment. The data present as mean ± S.E.M from three independent experiments (n=3). ***p<0.001 compared with untreated control.

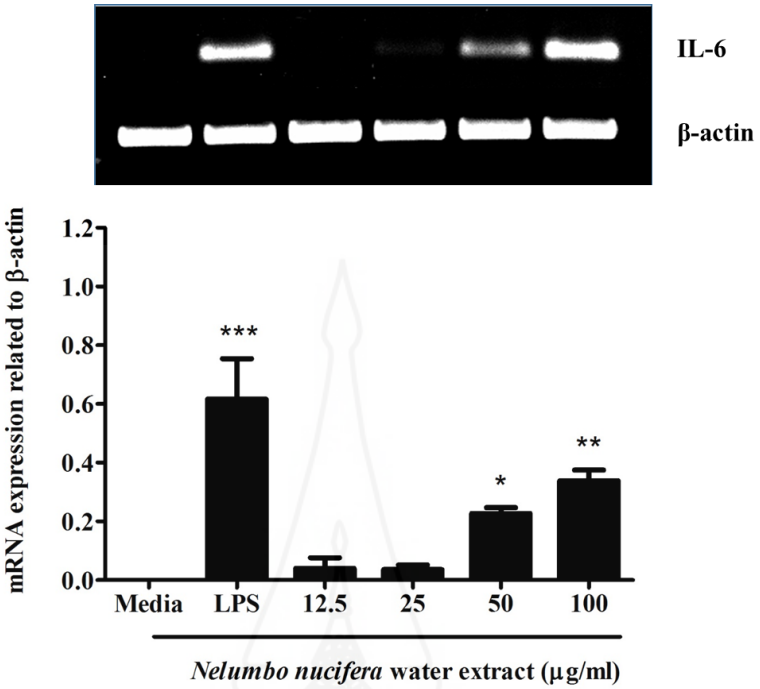

Figure 6. Effect of the water extract of *N. nucifera* at 12.5 – 100 μg/ml on mRNA expression of IL-6 in J774A.1 after 4 h of treatment. The data present as mean ± S.E.M from three independent experiments (n=3). *p<0.05, **p<0.01, ***p<0.001 compared with untreated control.

Discussion and Conclusion

Macrophages are essential cells which have multi-functions in innate and adaptive immunity. Any agents that activate macrophages may have medical benefit as immune enhancers for people who needed to improved immune responses such as patients with primary immunodeficiencies, radiation treated and chemotherapy treated or adjuvants in cancer therapy. In this study, we demonstrated that the water extract of lotus pollen from *N. nucifera* may have potential to be an immunostimulator. The extract increased NO production and iNOS mRNA expression in macrophage J774A.1 cells. NO is a free radical produced from L-arginine by iNOS, an inducible enzyme which does not express in resting macrophages but expresses in activated macrophages. iNOS induces large amount of NO which functions as toxic agent for destroying invaded pathogens in phagocytosis process. NO is also a mediator involves in inflammatory process in innate immunity.

Activated macrophages are sources of numerous cytokines and mediators which contribute to effective immune functions. The extract induced macrophage J774A.1 cells to express cytokines IL-1β, IL-6 and TNF-α. These cytokines play roles in inflammation, T cell activation, B cell matruration, recruitment of immune cells to infectious sites, and cytokine production in macrophages. They are often used as the markers of activated macrophages.

In conclusion, the results from this study reveal that the water extract of lotus pollen from *N. nucifera* has immunostimulatory effect on macrophages. The extract may have immune enhancer property in part by activating macrophages. Lotus pollen from *N. nucifera* may have potential to be used for boosting immune system in immunocompromised patients. However, this property need to be further studied *in vivo*.

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