

Anti-Inflammatory Potential of Ethanolic Bulb Extract of *Allium ascalonicum*

Nuttiya Werawattanachai^{1*}, Rawiwun Kaewamatawong¹, Jintana Junlatat², and Bung-orn Sripanidkulchai²

¹Faculty of Pharmaceutical Sciences, Ubon Ratchathani University,
85 Sathonlamark Rd., Warin Chamrap, Ubon Ratchathani 34190 Thailand

²Faculty of Pharmaceutical Sciences and Center for Research and Development of Herbal Health Products,
Khon Kaen University, Khon Kaen 40002 Thailand

*Email: phnuttw@mail2.ubu.ac.th

บทคัดย่อ

หอมแดง (*Allium ascalonicum* L.) เป็นเครื่องเทศและมีการนำมาใช้ในการแพทย์พื้นบ้าน การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ต้านการอักเสบของส่วนสกัดหัวหอมแดงในเอธานอลในหลอดทดลอง ทำการทดสอบความมีชีวิตรอดของเซลล์ด้วยวิธี 3-[4,5-dimethylthiazol-2-yl]-2,5-dyphenyl tetra-zolium bromide (MTT) ศึกษาผลของส่วนสกัดต่อการแสดงออกของยีนที่เป็นสื่อกลางการอักเสบ inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, COX-1, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β และ IL-6 ในเซลล์เพาะเลี้ยงมาโครฟาจ (RAW 264.7) ที่ได้รับการกระตุ้นด้วยสาร Lipopolysaccharide (LPS) โดยวัดปริมาณยีนที่แสดงออกด้วยวิธี reverse transcription polymerase chain reaction (RT-PCR) วิเคราะห์หาปริมาณฟีนอลรวมและฟลาโวนอยด์รวมของส่วนสกัดโดยใช้ปฏิกิริยาการเกิดสีกับสาร Folin-Ciocalteu และสารอลูมิเนียมคลอไรด์ตามลำดับ ผลการศึกษาพบว่าความเข้มข้น 62.5, 125 และ 250 ไมโครกรัม/มิลลิลิตร ส่วนสกัดหอมแดงในเอธานอลไม่มีความเป็นพิษต่อเซลล์และมีฤทธิ์ยับยั้งการแสดงออกของยีน iNOS, TNF- α , IL-1 β และ IL-6 เพิ่มขึ้นตามความเข้มข้น ส่วนสกัดหอมแดงไม่มีผลต่อการแสดงออกของยีน COX-2 แต่ยับยั้งการแสดงออกของยีน COX-1 อย่างมีนัยสำคัญ โดยมีปริมาณสารฟีนอลรวมคิดเป็น 15.964 ± 0.122 สมมูลกับกรดแกลลิก/กรัม และมีปริมาณสารฟลาโวนอยด์รวม 11.742 ± 0.012 มก.สมมูลกับสารเคอร์ซีทิน/กรัม จากผลการศึกษานี้ส่วนสกัดหอมแดงในเอธานอลมีศักยภาพที่จะนำมาพัฒนาเป็นยาด้านการอักเสบในอนาคต

คำสำคัญ : หอมแดง ฤทธิ์ต้านอักเสบ iNOS COX-2 สารสื่อกลางการอักเสบ

Abstract

Allium ascalonicum L. is used as a spice and has been used in traditional folk medicines. The anti-inflammatory effect of the ethanolic extract obtained from the bulbs of *Allium ascalonicum* L. (AAE) was evaluated in vitro. The effect of AAE on RAW264.7 cell viability was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-dyphenyl tetra-zolium bromide (MTT) assay. The effect of AAE on the expressions of inflammation associated genes, including inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, COX-1 tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6, in lipopolysaccharide (LPS)-stimulated RAW264.7 cells were determined by using reverse transcription polymerase chain reaction (RT-PCR) technique. Total phenolic content and total flavonoid content analyses were performed by Folin-Ciocalteu reaction and aluminium chloride colorimetric method, respectively. The nontoxic concentrations (62.5, 125, and 250 μ g/ml) of AAE were used for all experiments. The results indicated that the expressions of iNOS, TNF- α , IL-1 β , and IL-6 at the mRNA

level were inhibited by AAE in a concentration-dependent manner. AAE did not affect the COX-2 mRNA expression but significantly suppressed the COX-1 mRNA expression. The total phenolic compound of AAE was 15.964 ± 0.122 mg gallic acid equivalent. g^{-1} plant extract. Total flavonoid content of AAE was 11.742 ± 0.012 mg quercetin equivalent. g^{-1} plant extract. The present study revealed that the ethanolic extract of *Allium ascalonicum* contained constituents such as total phenol and total flavonoid contents and exhibited inhibitory effect against iNOS, and pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6, gene expressions in the LPS-stimulated RAW264.7 cells. Thus, *Allium ascalonicum* could be further developed to be an alternative anti-inflammatory agent.

Keywords: *Allium ascalonicum*: anti-inflammatory: iNOS, COX-2: pro-inflammatory cytokines

Introduction

Inflammation is a normal protective response to tissue injury caused by any noxious stimulus to destroy the invading organisms. Macrophages are the main pro-inflammatory cells responsible for invading pathogens by releasing inflammatory mediators such as nitric oxide (NO) produced by inducible NO synthase (iNOS), prostaglandins (PG) by cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6. Recent evidence has indicated that cytokines are involved in the pathophysiology of many inflammatory diseases, including sepsis, rheumatoid arthritis, atherosclerosis and asthma. Various compounds of plants show anti-inflammatory activity by inhibiting inducible nitric oxide synthase, cyclooxygenases or modulating the expressions level of inflammation-associated genes [1]. *Allium ascalonicum* is an important part of the diet of many populations and widely beneficial to health. To date, there has been report about the anti-inflammatory activity of *Allium ascalonicum* [2]. However, there is scant work regarding the detailed

mechanisms associated with anti-inflammatory action of *Allium ascalonicum*. Thus, in the present study we determined the effects of the ethanolic extract of *Allium ascalonicum* (AAE) on the expressions of inflammation-associated genes in the LPS-stimulated RAW264.7 cells

Materials and Methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin (Invitrogen, USA); *Escherichia coli* lipopolysaccharide (LPS), RNA extraction kit (GE Healthcare, UK); DNA ladder, Blue/Orange 6X loading dye (Promega, USA); iNOS, TNF- α , IL-1 β , IL-6, COX-2, COX-1, β -actin primers (Operon Biotechnologies, Germany); Agarose (Bio-Rad, Spain); RNase-free DNase Set, Omniscript reverse transcriptase kit, TopTaq MasterMix kit (Qiagen, Germany); RAW264.7 cells (PromoCell, Germany). All other chemicals were of analytical grade and were purchased from Sigma Chemicals (USA).

Preparation of the plant extract

The bulbs of *Allium ascalonicum* growing in Sisaket Province, Thailand were used as plant materials. They were cut into small pieces and macerated in ethanol for 3 days and filtered. The filtrate was evaporated under reduced pressure until dry and then was lyophilized. Murine macrophage cell culture: RAW264.7 cells were cultured in DMEM medium, containing 10% FBS and 1% penicillin-streptomycin and incubated at 37°C in humidified atmosphere of 5% CO₂ /95% air.

Cell viability assay

RAW264.7 cells (1x10⁴ cells/well) were treated with various concentrations of extract (0-500 µg/ml) and incubated at 37°C for 24 h. Cell viability was assessed by the MTT assay. The absorbance in each well was measured by using microplate reader at a wavelength of 570 nm. The results were calculated for % inhibition.

Detection of mRNA expressions of iNOS, COX-2, COX-1, TNF-α, IL-1β, IL-6 by reverse transcription polymerase chain reaction (RT-PCR) analysis

RAW264.7 cells (1x10⁶ cells/well) were overnight cultured and treated with various concentrations of extract. After incubation for 22 h, the LPS was added to a final concentration of 10 µg/ml and then further incubated for 2 h. Total RNA was purified from the cells using the RNA extraction kit according to the manufacturer's directions. The cDNA was synthesized from total RNA (40 ng) with Omniscript reverse transcriptase kit. Amplification was completed for 27

cycles by using a PCR thermal cycler (GeneAmp® PCR system 2400, Perkin-Elmer, USA) and the cycle conditions for PCR amplification were followed previous reports [3], [4]. The RT-PCR products were separated with 1.5% agarose gel electrophoresis and stained with Novel Juice and visualized under UV light. The density of target bands were measured by gel documentation and system analysis device (InGenius®, Bio-Rad, USA). Results were expressed as the relative mRNA expression values which were normalized with mRNA expression of β-actin.

Determination of total phenolic and total flavonoid contents

Total phenolic content was determined by using Folin-Ciocalteu method with gallic acid as standard. Total flavonoid content was determined by using aluminium chloride colorimetric method with quercetin as standard.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM) (n=3). The statistical significance of the difference between groups was evaluated by one way ANOVA followed by Least Significant Difference (p<0.05).

Results and Discussion

The effect of AAE on RAW264.7 cell viability

There were no significant inhibitory effects on cell survival with AAE at concentrations ranging from 62.5-250 µg/ml. However, AAE at a concentration of 500 µg/ml significantly decreased cell viability to

86% ($p < 0.05$). Influence of AAE on LPS-induced mRNA expressions: The expressions of inflammation-associated genes, including iNOS, COX-2, COX-1, TNF- α , IL-1 β , and IL-6 markedly increased following LPS stimulation (Fig 1A lane 3). Aminoguanidine and indomethacin, positive controls, significantly reduced the LPS-induced mRNA expressions of these inflammation-associated genes. Pretreating cells with AAE significantly decreased mRNA expressions of iNOS, COX-1, TNF- α , IL-1 β , and IL-6 in a concentration-dependent manner (Fig 1B, 1D, 1E, 1F, 1G, respectively). The expression of COX-2 was not significantly inhibited by AAE (Fig 1C).

Total phenolic and total flavonoid contents

The total phenolic compound of AAE was 15.964 ± 0.122 mg gallic acid equivalent. g^{-1} plant extract and the total flavonoid content of AAE was 11.742 ± 0.012 mg quercetin equivalent. g^{-1} plant extract.

The prolonged production of inflammatory mediators lead to many pathological states. iNOS, mainly responsible for the production of large amounts of NO, can elicit cellular toxicity which contribute to atherosclerosis and inflammatory diseases. TNF- α , IL-1 β , and IL-6 are primary cytokines involved in the progression of

chronic joint inflammation. Cyclooxygenase, which converts arachidonic acid to prostaglandins and eicosanoids, exists in two isoforms, COX-1 and COX-2. COX-2 is an inducible enzyme primarily expressed by cells that mediate inflammation. COX-1 is constitutively expressed in many tissues and mainly involved in housekeeping functions such as maintaining gastric mucosal integrity, initiating platelet aggregation, and regulating renal blood flow [1], [4]. In this study, AAE could be possible candidate to be considered in the treatment of inflammatory-mediated diseases since it reduced the productions of iNOS, TNF- α , IL-1 β , and IL-6 at the mRNA level. However, the anti-inflammatory activity of AAE might not directly through inhibiting COX-2 activity and it is possible that AAE could provoke some unwanted adverse effects by suppressing the COX-1 mRNA expression. Phenolic and flavonoid compounds have been used as nutritional supplements for their anti-inflammatory properties. The anti-inflammatory effects of AAE may be related to these compounds. However, it is still necessary to conduct detailed phytochemical studies of AAE to determine which active compounds might account for anti-inflammatory activity.

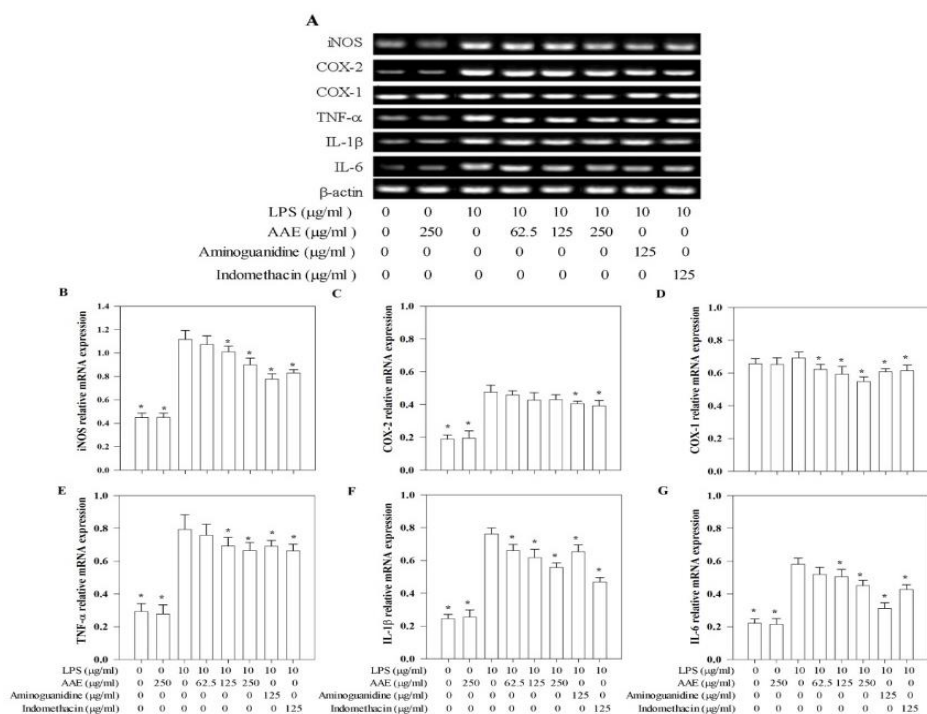


Figure 1 Inhibitory effects of AAE on the expressions of inflammation-associated genes in RAW264.7 cells (A) Amplified bands of iNOS, COX-2, COX-1, TNF- α , IL-1 β , IL-6, and β -actin of representative samples (B), (C), (D), (E), (F), and (G) the relative mRNA expressions of iNOS, COX-2, COX-1, TNF- α , IL-1 β , and IL-6, respectively, which were normalized to β -actin; *Significantly different from the LPS treatment alone at $p < 0.05$

Conclusion

In conclusion, the ethanolic extract of *Allium ascalonicum* contained total phenolic and total flavonoid compounds and exhibited inhibitory effect against iNOS, and pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6, gene expressions in the LPS-stimulated RAW264.7 cells. Thus, *Allium ascalonicum* could provide a beneficial effect for inflammatory-mediated diseases.

Acknowledgements

This study was financially supported by National Research Council of Thailand. The authors wish to thank Professor Koichi Ueno and Assistant Professor Hiromi Sato, Chiba University, Japan, for technical assistance in the preliminary study.

References

- [1] Calixto, J.B., Campos, M.M., Otuki, M.F., and Santos, A.R. 2004. "Anti-inflammatory compounds of plant origin. Part II. modulation of pro-inflammatory cytokines, chemokines and adhesion molecules". **Planta Med.** 70: 93-103.
- [2] Mohammadi-Motlagh, H.R., Mostafaie, A., and Mansouri, K. 2011. "Anticancer and anti-inflammatory activities of shallot (*Allium ascalonicum*) extract". **Arch Med Sci.** 7: 38-44.
- [3] Sugawara, I., Yamada, H., and Mizuno, S. 2003. "Relative importance of STAT4 in murine tuberculosis". **J Med Microbiol.** 52: 29-34.
- [4] Won, J.H., Im, H.T., Kim, Y.H., Yun, K.J., Park, H.J., Choi, J.W., Lee, K.T. 2006. "Anti-inflammatory effect of buddlejasaponin IV through the inhibition of iNOS and COX-2 expression in RAW264.7 macrophages via the NF-kappaB inactivation". **Br J Pharmacol.** 148: 216-225.