

ANTI-ALLERGY POTENTIAL OF PETIS EXTRACT ON IMMUNOGLOBULIN E PRODUCTION BY U266 CELLS

(Potensi Anti-alergi Ekstrak Petis terhadap Produksi Imunoglobulin E oleh Sel U266)

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ABSTRACT

Petis, one of Indonesia's traditional condiment, is most often made from shrimp and shrimp waste. This condiment becomes popular as a savory flavor additive which is mainly produced in East Java, Indonesia. Despite the unique taste and good nutritional content, any particular study regarding its health benefit in Indonesia has not thoroughly evaluated yet. Therefore, the objective of this research was to explore petis potential towards anti-allergy property *in vitro*. The potential property was evaluated by determining the concentration of Immunoglobulin E (IgE) by U266 cells treated with petis extract using ELISA. In result, petis extract could significantly suppressed IgE production up to 2-fold at its highest concentration compared to control. Further investigation to extrapolate petis functional bioactive compound was conducted by treating petis extract with heat and enzyme (proteinase K). Result showed that heat- and enzyme-treated petis extract still have the ability to suppress IgE production by U266 cells. Thus, it could be assumed that the functional bioactive compound was a heat-stable non-protein compound. These preliminary findings could conclude that petis extract has a potential anti-allergy property which gives an added value towards petis product in Indonesia.

Keywords: petis, IgE suppression, U266 cells, ELISA

ABSTRAK

*Petis merupakan salah satu kondimen tradisional Indonesia yang berbahan baku utama udang atau limbah udang. Kondimen ini terkenal sebagai penyedap rasa yang gurih dan banyak diproduksi di daerah Jawa Timur, Indonesia. Walaupun memiliki cita rasa yang khas dan kandungan gizi yang baik, sejauh ini belum ada kajian khusus mengenai khasiat petis untuk kesehatan di Indonesia. Oleh karena itu, eksperimen ini bertujuan untuk mengeksplorasi potensi anti-alergi ekstrak petis secara *in vitro*. Studi potensi tersebut dilakukan dengan mengevaluasi produksi Imunoglobulin E (IgE) oleh sel U266 menggunakan ELISA. Hasil penelitian menunjukkan bahwa ekstrak petis secara signifikan dapat menurunkan produksi IgE hingga 2 kali lipat pada konsentrasi tertinggi dibandingkan dengan kontrol. Untuk memprediksi senyawa bioaktif yang terkandung dalam ekstrak petis, maka dilakukanlah pengujian lanjut dengan memberikan perlakuan panas dan enzim (proteinase K) pada ekstrak petis. Hasil ELISA menunjukkan bahwa tetap terdapat tren penurunan produksi IgE oleh sel U266 meskipun ekstrak petis telah diberikan perlakuan panas dan proteinase K. Hal ini diperkirakan bahwa senyawa bioaktif tersebut merupakan komponen selain protein yang bersifat stabil pada suhu tinggi. Penemuan awal ini dapat menyimpulkan bahwa ekstrak petis memiliki potensi anti-alergi yang dapat diperkenalkan sebagai nilai tambah bagi produk petis di Indonesia.*

Kata kunci: petis, penurunan produksi IgE, sel U266, ELISA

I. INTRODUCTION

Petis, one of Indonesia's traditional condiment, is mainly produced from shrimp and its waste including shrimp skin, head, and tail. This condiment is popular with its unique characteristics: coal-black, sticky, pliant, and savory. The production of petis in Indonesia, especially in East Java, has been developed up to industrial scale (Huda, 2012). Commonly, the process of making petis starts with boiling the smashed shrimp waste along with the addition of sugar or palm sugar. After the solution is brought to boil, flavoring such as salt is added. Other than bringing a tasteful flavor to the petis, high salt content naturally acts as the preservative to the final product. Therefore, petis have a long shelf life (Jurusan Teknologi Pangan dan Gizi-IPB, n.d.).

The nutritional content in 100 gram of shrimp petis according to the data obtained from Daftar Komposisi Bahan Makanan (DKBM) (Depkes, 2016) is as follows: 220 kcal energy, 15 gr protein, 0.1 gr fat, 40 gr carbohydrate, 37 mg calcium, 36 mg phosphorus, and 3 mg iron. Even though petis contains nutritious macro- and micronutrients, study regarding the effect of petis consumption towards health is rarely conducted. The latest evaluation found out the beneficial fibrolytic enzyme contained in petis which can be a therapeutic food for curing cardiovascular disease (Mine, Kwan Wong, & Jiang, 2005). Other than that, the knowledge of petis has not explored yet in Indonesia. So, heretofore, the reason to consume petis is only based on its desired sensorial properties.

The objective of this study was to evaluate the effect of petis extract towards immune cells *in vitro*. By discovering novel health potential of petis, it is expected that the selling points and value of petis may increase. Thus, introducing petis as one of the valuable functional fermented food in Indonesia.

The cells used in this study were U266 cells which are a multiple myeloma secreting immunoglobulin E from peripheral blood (Sugahara, *et al.*, 2009). Immunoglobulin E (IgE) is an antibody which induces type 1 hypersensitivity (Gould *et al.*, 2003). The excessive signal for IgE secretion towards allergens can cause various allergic symptoms for type 1 hypersensitivity patients.

The evaluation of petis extract effect towards IgE production by U266 cells was conducted using enzyme-linked immunosorbent assay (ELISA). In addition, petis extract was further treated by heat and enzyme (Proteinase k) in order to predict the functional bioactive compound in petis.

II. METHODOLOGY

2.1 Sample preparation

Commercially available petis (Petis Udang) was bought from the local supermarket in Jakarta area. It was dissolved in deionized water at 0.1 gr mL^{-1} and mixed for 24 h at 4°C . The solution was then centrifuged (12,000 rpm, 20 minutes, 4°C). The supernatant was collected and further centrifuged (70,000 rpm, 30 minutes, 4°C). The supernatant was collected again and filtered through $3 \mu\text{m}$ cellulose acetate membrane. The obtained filtrate was dialysed in a 500 Da dialysis membrane for 48 h at 4°C . The dialysed solution was collected and the pH was adjusted to 7.4. The final solution was sterilized by a $0.22 \mu\text{m}$ filter, and collected into microtubes. All petis extract samples (PES) were stored at -35°C prior to analysis.

2.2 Sample treatments

2.2.1 Heat treatment

Some prepared PES were heated at 100°C for 30 minutes. Then, they were cooled down to room temperature. Samples were centrifuged at 3,500 rpm, 4°C for 10 minutes. The supernatant was collected into new 1.5

mL microtubes and stored in -35°C prior to analysis.

2.2.2 Enzyme treatment

Ten milligrams of Proteinase K was diluted with 2 mL of deionized water and mixed for 15 minutes. The solution was filtered using a $0.45\ \mu\text{m}$ filter aseptically. Proteinase K solution was added to some prepared PES at $100\ \mu\text{L}$ each. The samples were incubated at 37°C for 20 hours. Proteinase K activity was deactivated by heating at 100°C for 5 minutes. Samples were centrifuged at 3,500 rpm, 4°C for 10 minutes. The supernatant was collected into new 1.5 mL microtubes and stored in -35°C prior to analysis.

2.3 Total protein quantification

Protein standard BSA (bovine serum albumin) solutions were prepared at the following concentrations adjusted with phosphate buffered saline (PBS): 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, and $2000\ \mu\text{g mL}^{-1}$. Five microliters of each sample and standard solution was transferred into their respective wells in a 96-microwells plate. Then, $25\ \mu\text{L}$ of Reagent A (BIORAD) was added to each wells, followed with the addition of $200\ \mu\text{L}$ Reagent B (BIORAD) away from light. The plate was shaken with a mixer for 15 minutes. The absorbance of sample was read using plate reader with the setting as follows: measure = $655\ \text{nm}$ and reference = $415\ \text{nm}$.

2.4 ELISA

2.4.1 U266 cell culture

PES were diluted to several concentrations: 75, 300, and $675\ \mu\text{g mL}^{-1}$. Then, $50\ \mu\text{L}$ of each sample was transferred to each respective well in a 96-microwells plate. Ten millimolars of NaPB was used as the control. Medium solution for U266 cells consists of 10 mL sterilized water, $100\ \mu\text{L}$ Insulin (Sigma), $100\ \mu\text{L}$ Transferrin (Sigma),

$100\ \mu\text{L}$ Ethanolamine (Sigma), and $100\ \mu\text{L}$ Sodium Selenite (Sigma). Fifty microliters of medium solution was transferred into each respective wells in a 96-microwells plate. Then, U266 cells maintained in a $1\times$ RPMI-1640 medium were seeded into a 96-well culture plate (Corning) at 0.5×10^4 cells well^{-1} . The sample and cells were incubated at 37°C with 5% CO_2 for 20 hours before conducting ELISA assay.

2.4.2 IgE ELISA

Coating: $100\ \mu\text{L}$ of $1000\times$ diluted anti-human IgE antibody in a 50 mM carbonate buffer (pH 9.6) was transferred into each respective wells in a 96-microwells plate. The plate was incubated at 37°C for 2 hours.

Blocking: the plate was washed three times with Tween20-phosphate buffered saline (T-PBS). Then each wells was filled with $300\ \mu\text{L}$ 5% skimmed milk-PBS solution. The plate was incubated at 37°C for another 2 hours.

Sampling: 5% skimmed milk-PBS and $100\ \text{ng mL}^{-1}$ IgE standard solution was prepared at the following concentrations: 0, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, and $100\ \text{ng mL}^{-1}$. The incubated plate was washed three times with T-PBS. Then each respective wells was filled with $50\ \mu\text{L}$ standard solution or the supernatant of cultured U266 cells. The plate was incubated at 37°C for 1 hour.

2nd Antibody: the plate was washed three times with T-PBS. Then each wells was filled with $100\ \mu\text{L}$ anti-human IgE 2nd antibody (Biosource International AHI0604) which has been $20000\times$ diluted with 5% skimmed milk-PBS. The plate was incubated at 37°C for another 1 hour.

Avidine-Biotin Complex: the plate was washed three times with T-PBS. One hundred microliters of conjugated streptavidin-horseradish peroxidase (diluted $4000\times$ with 1% BSA-PBS) was added to each respective well. The plate was incubated at 37°C for 1 hour.

Coloring: the plate was washed three times with T-PBS. A coloring solution consists of citrate buffer (pH 4.0),

deionized water, and 0.06 mg mL⁻¹ ABTS with the ratio of 10:9:1 was prepared. One hundred microliters of the coloring reagent was transferred into each respective well. The plate was let to sit for 10 minutes away from light. One hundred microliters of stop solution consisting of 1.5% oxalate acid was added to each well. The sample absorbance was read using plate reader with the setting as follows: measure = 415 nm and reference = 655 nm.

2.5 Real-time RT-PCR

U266 cells suspended in ITES-RPMI-1640 medium (1.25 mL) containing PES at 675 µg mL⁻¹ (1.25 mL) were seeded into a 60 × 15 mm culture dish at 5 × 10⁵ cells dish⁻¹. After incubation for 24 h at 37°C, cells were harvested and RNA was isolated using Sepasol-RNA I Super G (Nacalai Tesque) according to the manufacturer's instruction to obtain cDNA template using MMLV-reverse transcriptase (Promega, Madison, WI, USA) and oligo-(dT)₂₀ (Toyobo).

Table 1. Primer sequences

Genes	Sequences
Human IgE	5'-ATGACCTTACCAGCCACC AC-3' (sense) 5'-GGTTTTGTTGTCGACCCA GT-3' (anti-sense)
Human GAPDH	5'-TCCATGACAACCTTGGCATCGT GG-3' (sense) 5'-GTTGCTGTTGAAGTCACAGGAG AC-3' (anti-sense)

Real Time RT-PCR samples consisting of 2 µL of cDNA sample, 10 µL of SYBR Green PCR Master Mix, 1 µL each of 10 µM forward and reverse primer were prepared in microtubes. Thermal cycling conditions were prepared with the following settings: 95°C for 20 seconds, 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds. The relative gene expression was calculated based on the comparative CT method and normalized by measuring in parallel approach to the

expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Primer sequences used in this experiment are shown in **Table 1**.

2.6 Statistical calculation

Data obtained were expressed as mean ± standard deviation (SD). One-way ANOVA followed by student's t-test was used to assess the statistical significance of the difference against control. Values with * *p* < 0.05, ** *p* < 0.01, or *** *p* < 0.001 were considered statistically significant.

III. RESULTS AND DISCUSSION

3.1 IgE Suppression and Mechanism

IgE is produced and secreted by the plasma cells after an invading allergen was detected. IgE will attach to the surface of mastocyte; thus, sending signals to secrete histamine and cytokines. People who suffer from type I hypersensitivity overproduce IgE towards the detected allergen. Therefore, the secretion of histamine and cytokines become excessive in blood and result in allergic reaction which symptoms may vary for every patients (Gould, et al., 2003).

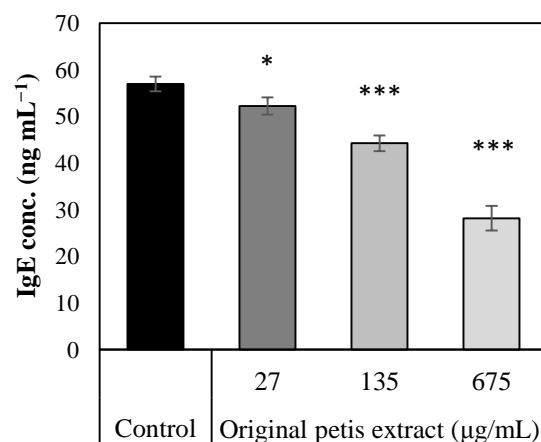


Figure 1. Effect of petis extract on IgE production by U266 cells. Results are shown as mean ± standard deviation of two

independent measurements. Statistically significant differences against control are represented as * $p < 0.05$ or *** $p < 0.001$.

According to IgE ELISA result shown in **Figure 1**, it was found that petis extract could significantly suppressed the production of IgE by U266 cells at increasing concentration. The suppression of IgE production reached up to 2-folds at the highest concentration compared to that of the control.

Correlating this finding to the existing molecular pathway, the suppression of IgE production may decrease the secretion signal for histamine and cytokines. Therefore, the allergic symptoms for type 1 hypersensitive patients can be reduced. This result proposed a novel potential of petis extract in having an anti-allergy property.

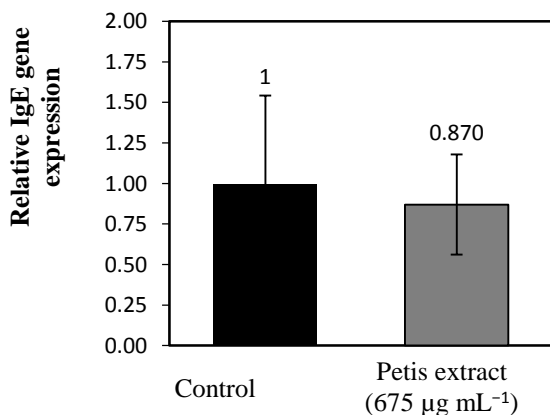


Figure 2. Effect of petis extract on IgE gene expression by U266 cells. Results are shown as mean \pm standard deviation of two independent measurements.

Subsequently, real time RT-PCR was conducted to further extrapolate the possible IgE suppressing mechanism of petis extract. As shown in **Figure 2**, IgE relative gene expression level between control (1.00) and petis extract-treated U266 cells (0.87) were not significantly different. This result found that IgE suppressing mechanism of petis extract was not through lowering IgE gene expression level of the cells. Further analysis

in translational, post-translational, and/or other protein turnover mechanisms should be conducted to determine the other possible IgE suppressing mechanisms of petis extract (Welle, 1999).

3.2 Initial extrapolation of IgE suppressing bioactive compound

The bioactive compound in petis was extrapolated by giving some treatments towards petis extract: heat and enzyme treatments. Heat treatment was used to denature all heat labile compounds contained in petis extract prior to ELISA, leaving only heat stable compounds functioning. The purpose of adding Proteinase K in enzyme treatment was to break the peptides bonds within the sample. Therefore, proteins were denatured prior to ELISA.

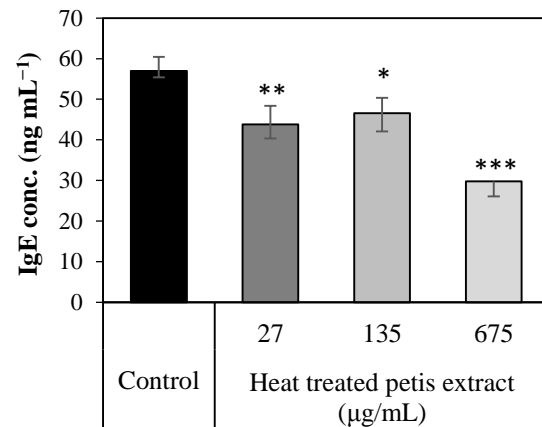


Figure 3. Effect of heat-treated petis extract on IgE production by U266 cells. Results are shown as mean \pm standard deviation of two independent measurements. Statistically significant differences against control are represented as * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

Heat-treated petis extract still showed a lowering IgE production effect compared to that of the control (**Figure 3**). Moreover, enzyme-treated petis extract (**Figure 4**) also showed the same suppressing trend as the untreated petis extract (**Figure 1**) despite the distinctive significant difference value. The

results from heat and enzyme treatment proposed that the properties of the bioactive compound contained in petis extract was heat stable and assumed to be a non-protein compound.

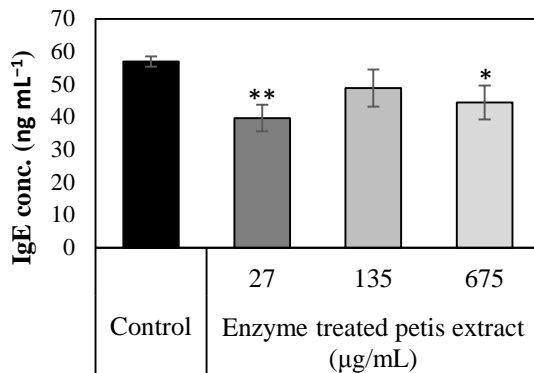


Figure 4. Effect of enzyme-treated petis extract on IgE production by U266 cells. Asterics annotating level of significant difference compared to control (* $p < 0.05$ and ** $p < 0.01$).

IV. CONCLUSION

Petis extract performed a novel potential of anti-allergy property through its significant suppression of IgE production *in vitro* compared to that of the control sample. Its mechanism, however, was not occurred through alteration of IgE gene expression level, but rather through other possible mechanisms that should be further researched. Result from heat and enzyme treated petis extract assumed that the functional bioactive compound contained in petis extract was a heat-stable non-protein compound. The findings of this study could contribute as preliminary data in developing IgE suppressing functional food for type I hypersensitive patients.

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