

Short Communication:

IgE production-suppressing effect of *asam kandis* (dried *Garcinia xanthochymus*) extracts by mouse hybridoma aDNP-Hy Cells

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Abstract. Supardi N, Sumarpo A, Sutejo R, Putra ABN, Kageyama N, Kawano M, Sugahara T. 2018. Short Communication: IgE production-suppressing effect of *asam kandis* (dried *Garcinia xanthochymus*) extracts by mouse hybridoma aDNP-Hy Cells. *Biodiversitas* 19: 330-335. *Asam kandis* (dried *Garcinia xanthochymus* Hook.f.ex.T.Anderson) is popularly used as a spice in many Indonesian cuisines. Although *Garcinia xanthochymus* has been reported to possess various pharmacological properties, its immunologic functions have been poorly studied. Therefore, in this study, we aimed to determine immunoglobulin E (IgE) production-suppressing effect of *Asam Kandis* extract (AKE) using mouse hybridoma aDNP-Hy cells. AKE was prepared through 24 hours maceration in distilled water. IgE production-suppressing activity of AKE by aDNP-Hy cells was examined in time-wise manner. Cell viability and IgE mRNA expression were assessed using trypan blue assay and qRT-PCR, respectively. IgE production-suppressing activity of heat-treated (100°C) AKE was also examined. As a result, AKE significantly reduced IgE production by aDNP-Hy cells in a concentration-dependent manner both in 24 and 36 h incubation periods. Furthermore, AKE exhibited its IgE production-suppressing activity without any cytotoxicity and also significantly decreased IgE mRNA expression. Heat-treated AKE showed no IgE production-suppressing activity, indicating that the bioactive substances are heat labile. More interestingly, heat-treated AKE showed cytotoxic effect by aDNP-Hy cells. We showed that AKE exhibits IgE production-suppressing effect by aDNP-Hy cells. Further studies are necessary to discover underlying bioactive compounds of *asam kandis* and substance that responsible for the cytotoxic effect of heat-treated AKE.

Keywords: *asam kandis*, *Garcinia xanthochymus*, IgE

Abbreviations: AKE = asam kandis extract; BSA = bovine serum albumin; DEPC = diethyl pyrocarbonate; FBS = fetal bovine serum; GADPH = glyceraldehyde-3-phosphate dehydrogenase; IgE = immunoglobulin E; IL-4 = interleukin 4; PAF = platelet aggregating factor; PBS = phosphate-buffered saline; PGD2 = prostaglandin D2; RPMI 1640 = Rosewell Park Memorial Institute 1640; TNF = tumor necrosis factor; T-PBS = tween 20 phosphate-buffered saline

INTRODUCTION

Allergy has been one of the world's serious problems in the last decade. Allergy is a hypersensitive reaction that is mediated by immune system of the body (Pudjiadi et al. 2009). Immunoglobulin E (IgE), the antibody component of adaptive immune system that has the capability to destroy parasites, has been long found to be responsible for this unfavorable reaction (Burton and Oettgen 2011). The IgE binds to a relatively harmless antigen and this crosslinking eventually causes allergic reaction that occur within minutes (Burton and Oettgen 2011). Thus, people that possess allergy have higher serum IgE in comparison to healthy individuals. The most common manifestations of this reaction include asthma, allergic rhinitis, conjunctivitis, gastroenteritis, and also atopic dermatitis (Burton and Oettgen 2011; Bellik et al. 2012).

Prevalence of allergy is rapidly increasing every year, in both developing and developed countries around the

world. According to a report by World Allergy Organization (WAO) in its book *WAO White Book on Allergy*, which is published in 2013, approximately 30-40% of the whole world population is now being affected by allergy and this number keeps on rising annually (WAO 2013). Most of clinical cases of allergy, including in Indonesia, rely on the drug medications to suppress the reaction. Nevertheless, this might, in long-term use, elicits side effects. Due to this matter, the demand for foods with anti-allergic effect that may lower down IgE production constantly attracts the interest of people. This is due to definitely lower side effects compared to drugs and therefore, more scientific evidences for this kind of foods are needed.

Indonesia is very rich in its cultures and biodiversity. Through this, many cuisines were created as the reflections of each culture's identity. Most of the cuisines utilize own biodiversity that has been noticed to be extremely abundant for centuries even by the ancestors. One of the most

prominent biodiversity in Indonesian cuisines is spice. Spices have been identically linked and become one of the essential parts to most of Indonesian cuisines. These spices contribute to the colorful and luscious flavors of the dishes, making them recognizable to the world. In the recent years, many studies have explored more in regards to Indonesian biodiversity, including the spices, as they have been associated with some biological activities believed since the ancient medication.

Asam kandis is a spice that is commonly used in the cuisines of Sumatra Island of Indonesia. It is derived from the skin of *Garcinia xanthocymus* Hook.f.ex.T.Anderson fruit that is sun-dried until it becomes black-red in color (Ismawan et al. 2012). *G. xanthocymus* has been utilized long ago as medicine for skin infection, diarrhea, dysentery, and also wound (Heyne, 1987; Boggett et al. 2005). Some studies have demonstrated the biological potential of *G. xanthocymus*, including antioxidant (Zhong et al. 2009), anti-toxicity (Han et al. 2007), anti-inflammation (Pal 2005; Hamidon et al. 2009), cytotoxic effect (Hamidon et al. 2009), potential neuron growth factor (Chanmahasathien et al. 2003), and anti-malarial properties (Indarti et al. 2009).

Nonetheless, despite of these promising biological activities of *G. xanthocymus*, all of these studies are limited to the bark and leaves of the *G. xanthocymus*, while the dried skin or spice form remains elusive. In addition, its capacity in the immunological point of view has been poorly demonstrated, especially in the IgE-suppressing activities. Therefore, our study was aimed to determine the IgE-suppressing effect of AKE *in vitro* by aDNP-Hy cells.

MATERIALS AND METHODS

Procedures

Reagents

Roswell Park Memorial Institute 1640 (RPMI 1640) medium, fetal bovine serum (FBS), and bovine serum albumin (BSA) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat anti-mouse IgE antibody and biotin-conjugated rat anti-mouse IgE were products of BD Pharmingen (San Diego, CA, USA). HRP-labeled streptavidin and 2,2'-azinobis (3-ethylbenzo thiazoline-6-sulfonic acid ammonium salt) were from Wako Pure Chemical Industries (Osaka, Japan).

Sample preparation

Asam kandis (dried *G. xanthocymus*) was purchased from Indonesian local market located in Sunter, North Jakarta, Indonesia. Prior the extraction, *asam kandis* was grinded using miller until it was finely grounded and stored at -18°C freezer. It was macerated in distilled water (0.1 g/mL) at 12°C for 24 h on a rotator (15 r/min) and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected and further centrifuged at 70,000 rpm for 20 min at 4°C. The supernatant was taken and filtered (0.45 µm) before processed to another step. The pH value of the supernatant was adjusted to pH 7.4. It was then filtered (0.22 µm) and stored at -35°C freezer before

further usage. The extract was named as *asam kandis* extract (AKE) in this experiment. Moreover, in order to investigate the heat stability of the sample, the AKE was heated on heat block at 100°C for 10, 30, 60 min and stored at -35°C before further assessment.

Protein quantification

Protein quantification was done in order to determine the protein concentrations of AKE. It was assessed using DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with purified BSA as standard according to manufacturer's instructions.

Cell culture

Mouse hybridoma aDNP-Hy cells were gift from Dr. Kazutaka Maeyama, Faculty of Medicine, Ehime University. The aDNP-Hy cells are hybridoma cells that constantly produce IgE. These aDNP-Hy cells were cultured in RPMI 1640 medium that has been supplemented with 10% FBS in 100 mm culture dish (BD Falcon, Franklin Lakes, NJ, USA) at 37°C under humidified 5% CO₂.

IgE production-suppressing activity

The aDNP-Hy cells were suspended in RPMI 1640 medium supplemented with 10% FBS and various concentrations of AKE. They were seeded with seeding density of 1.0×10^5 cells/well in 96-well culture plate (BD Falcon) and incubated at 37°C under humidified 5% CO₂. After 12, 24, and 36 h of incubation periods, the IgE level was quantified using ELISA and the remaining cells or supernatant were used for cytotoxic assay, which all referred to the next following sections.

Enzyme-linked Immunosorbent Assay (ELISA)

The IgE concentration in the culture media was measured by an in-house-developed ELISA. The 96-well microtiter plate (Nunc, Roskilde, Denmark) was coated with 100 µL of rat anti-mouse IgE antibody at 0.5 µg/mL concentration in PBS and incubated overnight at 4°C. Following the coating, washing was done with 0.05% T-PBS 3 times and each well was blocked with PBS that contained 1% (w/v) BSA (300 µL) for 30 min at room temperature. After washing with T-PBS 3 times, culture media (50 µL) and mouse IgE (50 µL) as standard were added to the well and incubated for 1 h at room temperature. Following washing with T-PBS 3 more times, each well was incubated with 100 µL biotin-conjugated rat anti-mouse IgE diluted in 0.5 µg/mL in 1% BSA-PBS for 1 h at room temperature. The plate was washed 6 times and treated with HRP-labeled streptavidin (100 µL) diluted at 1.25 µg/mL in 1% BSA-PBS for 30 min at room temperature. After washing 6 times with T-PBS, 100 µL of 0.6 mg/mL 2,2'-azinobis (3-ethylbenzo thiazoline-6-sulfonic acid ammonium salt) dissolved in 0.03% H₂O₂-0.05 M Citrate Buffer (pH 4.0). After color development, 1.5% oxalic acid (100 µL) was added to the plate. The absorbance was measured using Model 550 microplate reader (Bio-Rad) at 415 nm (reference: 655nm).

Cell viability assay

Cell viability assay that was used in this experiment was trypan blue exclusion assay. The cell solution was centrifuged at 35,000 rpm for 10 min at 4°C. Following the centrifugation process, the supernatant was removed and the cells were suspended in new medium (50 µL). The cell suspension (30 µL) were then taken, mixed with trypan blue (10 µL), and incubated in room temperature for 10 min. The numbers of dead and total cells were counted manually by preparing the mixture (10 µL) in the hemocytometer under the microscope.

RNA extraction

The cells were seeded and incubated as described in the "IgE production-suppressing assay" section for 30 h. The culture medium (150 µL) was retrieved and added with PBS (150 µL). This suspension was centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was removed and the PBS washing was repeated one more time. After the supernatant was decanted, total RNA was isolated by using 1 mL of Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and incubated for 5 min at room temperature. Chloroform (200 µL) was then put in, mixed vigorously, set aside in room temperature for 3 min, and centrifuged at 12,000 rpm for 15 min at 4°C. The clear upper part (500 µL) was collected, added with 2-propanol (500 µL), incubated in room temperature for 10 min, and centrifuged again at 12,000 rpm for 15 min at 4°C. Following the centrifugation process, the supernatant was removed, added with 75% ethanol (1 mL), mixed gently, and further centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was removed and the remaining ethanol was left air-dried for 10 min. After air-dried process, 10 µL of diethyl pyrocarbonate (DEPC) was added. The concentration and purity of RNA was measured by using UV/VIS (purity: 260/280 ratio in range of 1.8 to 2) before further assessment.

qRT-PCR

The cDNA synthesis was done through the RT-PCR of the isolated RNA. The RNA (1 µg) was added with DEPC water until the volume reach 13.5 µL and further supplied with 0.5 µL of 10 mM Oligo (dT)₂₀ primer (Toyobo, Osaka, Japan). The mixture was heated at 70°C for 10 min in the thermal cycler. Following the heating, 2.5 mM dNTP (5 µL), 5x Buffer (5 µL), RNase inhibitor (0.5 µL), DEPC water (0.3 µL) and MMLV-Reverse Transcriptase (Promega, Madison, WI, USA) (0.2 µL) were added to the heated mixture and further reacted at 37°C for 60 min in the thermal cycler. The cDNA was then undergone qPCR to quantify the mRNA level of IgE. The cDNA (10 µg) was added with Thunderbird SYBR qPCR mix (Toyobo) (10 µL), 10 µM forward primer (1 µL), 10 µM reverse primer (1 µL), and ultrapure water (6 µL). The thermal cycling conditions were 95°C for 10 min, 40 cycles of 3 s at 95°C and 30 s at 60°C, continued with 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The quantification was done by StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the relative gene expression was calculated automatically based on the

comparative CT method using StepOne software v.2.1 (Applied Biosystems). The gene expression was normalized by using the expression of house keeping gene: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the same sample. Specific primer sequences for mouse IgE: sense, 5'-ATGACCTTACCAG-CCACCAC-3' and anti-sense, 5'-GGTTTTGTTGTCGACCCAGT-3'.

Data analysis

The data obtained from this experiment were shown in mean ± standard deviation (SD). One-way ANOVA test followed with Tukey-Kramer test were utilized in order to determine the statistical significance of difference. The value of * $p < 0.05$ and ** $p < 0.01$ were considered as statistically significant.

RESULTS AND DISCUSSION

Effect of AKE on IgE production by mouse hybridoma aDNP-Hy cells

The aDNP-Hy cells were cultured in RPMI 1640 medium containing various concentrations of AKE in 3 incubation periods, which were 12, 24, and 36 h. The IgE concentration was then measured with ELISA method. This was done in order to determine and reassure the effect of AKE on IgE production by aDNP-Hy and also to know the best incubation time for AKE to show the most effective IgE-suppressing activity. As shown in Figure 1A, AKE significantly reduced IgE production in a concentration-dependent manner at both 24 and 36 h incubation periods. Nevertheless, in the 36 h, more significant decrease (t -test, ** $p < 0.01$ in all concentrations) was observed compared to 24 h incubation period (t -test, both * $p < 0.05$ and ** $p < 0.01$). IgE production was even suppressed for more than 50% when treated with AKE in 219 µg/mL concentration for 36 h.

Furthermore, the survival rate of aDNP-Hy cells, which have been exposed to various concentrations of AKE for 24 h, was determined by using trypan blue exclusion assay. The stained total and dead cells were measured and determined by utilizing hemocytometer under the microscope. This was done in order to ensure that AKE exhibited its IgE production-suppressing activity without cytotoxicity. There was no cytotoxicity observed in all concentrations of AKE (Figure 1B) since the survival rates of aDNP-Hy are all beyond 90%.

Effect of AKE by mouse hybridoma aDNP-Hy IgE mRNA expression

The IgE-suppressing activity of AKE was also observed at molecular level. The level of IgE mRNA relative expression by aDNP-Hy was determined by using the qRT-PCR method. The aDNP-Hy cells were cultured in RPMI 1640 medium and exposed to the highest and lowest concentration of the effective AKE concentrations, which were 55 and 219 µg/mL. The cells were incubated for 30 h prior qRT-PCR, remembering the fact that the mRNA is usually expressed before it is translated to protein (referring 36 h as the best time for IgE protein expression). Figure

1.C shows that the relative IgE mRNA level was significantly decreased in both concentrations (*t*-test, ** $p < 0.01$) compared to control.

Effect of heat-treated AKE on IgE production by mouse hybridoma aDNP-Hy

The AKE has been shown to possess IgE-suppressing activity towards aDNP-Hy cells. Nonetheless, *asam kandis*, as spices, is required to be heated as cooking ingredients. Therefore, the effect of heat-treated AKE was also observed in this study in order to know whether the IgE-suppressing activity still occurs following exposure of heat and at the same time, the heat-stability of the bioactive substances responsible for this effect can also be determined. The aDNP-Hy cells were exposed to various concentrations of AKE that have been previously heated in the thermal block at 100°C for 10, 30, and 60 min. The IgE concentration was then measured using ELISA. The heat-treated AKE showed no significant decrease in the IgE production by aDNP-Hy cells after 36 h of exposure (Figure 2). In addition, heat-treated AKE elicited cytotoxic effect at the highest concentration, which is 219 µg/mL

Discussion

IgE mediates type I hypersensitive reaction, which is commonly called as immediate hypersensitivity. This term represents the extremely sensitive nature of the IgE itself and also the exceptional speed of this reaction (Burton and Oettgen, 2011). In the presence of antigen, B cells undergo irreversible commitment and differentiate further to produce IgE antibodies (Talay et al. 2012). The IgE primarily binds to the FcεR1, CD 23, and other receptors that present in the surface of mast cells, basophils, and a wide range of tissues in the body (Gould and Sutton, 2008; Burton and Oettgen, 2011; Galli and Tsai, 2012). This crosslinking event leads to the activation and degranulation of mast cells, resulting in the release of several preformed and newly synthesized inflammatory mediators, such as interleukin 4 (IL-4), tumor necrosis factor (TNF), histamine (5-HT), serotonin, platelet aggregating factor (PAF), prostaglandin D2 (PGD2), and other variety of cytokines (Burton and Oettgen, 2011; Platzer et al. 2011; Bellik et al. 2012). All of these molecules causes the symptoms of immediate hypersensitivity reaction in allergic people (Burton and Oettgen, 2011). Therefore, many therapeutic approaches for allergic reaction focus on the suppression of IgE production in order to regulate the inflammatory mediators that elicit all unfavorable symptoms (Bellik et al. 2012; Holgate, 2014).

Previously, we screened several Indonesian spices extracts: cardamom (*Amomum compactum*) seed, *asam kandis* (dried *G. xanthocymus*), and candlenut (*Aleurites moluccana*) seed extracts for IgE-suppressing activity by aDNP-Hy cells. It was demonstrated that AKE exhibited the most potential IgE suppressing activity compared to other spices (data not shown). Therefore, in this study, we focused on the AKE and further assessed it as an anti-allergic functional food candidate.

In this study, we showed that AKE reduces IgE production by aDNP-Hy cells in a concentration-dependent

manner. Thus, this indicates that AKE is a very promising candidate for anti-allergic functional food candidate, linking to the fact that people with allergy have high serum IgE level. Moreover, this study also demonstrated that both 24 and 36 h incubation periods reduced IgE production significantly. Nevertheless, it is clear that 36 h of AKE exposure was the best time for AKE to elicit its IgE production-suppressing activity, even when compared to 24 h. 12 h incubation period is the least favorable exposure time since it did not show any significant reduction of IgE production. Therefore, according to the time periods done in this experiment, 12 h of AKE exposure is not enough to stimulate its IgE production-suppressing activity and it needs at least 24 h of incubation period to elicit a significant reduction in all concentrations. 36 h incubation time might be utilized as a reference of AKE exposure for further studies. In addition, our data also suggested that AKE induced IgE production-suppressing activity without any cytotoxicity, which seen from the high survival rate of the aDNP-Hy cells (Figure 1B). This shows that AKE surely exhibited IgE production-suppressing activity and has one more desirable characteristics as anti-allergic functional food candidate. Furthermore, the level of IgE mRNA relative expression was observed to be significantly decreased in this study. The reduced trend was also in line with the IgE production, which was in a concentration-dependent manner. This demonstrates that AKE affects the cell molecular level and brings out its IgE production-suppressing activity through the lower down of IgE gene expression.

For the heat-treated AKE, our result indicated that it did not elicit any significant reduction in the IgE production by aDNP-Hy cells (Figure 2). This result is actually contradicted with the untreated AKE sample that has been demonstrated previously and therefore, it indicates that heat treatment of AKE causes loss of IgE production-suppressing activity. It also clarifies that the bioactive substance that responsible for IgE-suppressing activity within AKE is heat unstable since the IgE production-suppressing activity was not observed following the heat exposure. More interestingly, the highest concentrations in all heat treatment periods were shown to be cytotoxic to the cells, whereas, this concentration was not cytotoxic in untreated AKE. This shows that heat exposure towards AKE might cause some changes in the substances that cause or strengthen the cytotoxic effect of the AKE towards the cells. Some studies have observed the effect of heat treatment on functional foods in relation to its cytotoxic effect. Heat treatment mostly leads to the conformational changes in the protein and lipid. This heat treatment can actually increase or decrease the cytotoxicity of the substance based on the changes it encounters. For instance, Park et al (1999) reported that the heat-treated Korean mistletoe showed lower cytotoxic effect against cancer cell, meanwhile study by Leclere et al. (2016) found out that heat treatment of citrus pectin cause significant increase in its cytotoxicity against cancer cells (Park et al. 1999; Leclere et al. 2016). Therefore, it is very important to investigate the cytotoxic substance of AKE in the future study since *asam kandis* utilization always involves heat exposure during cooking.

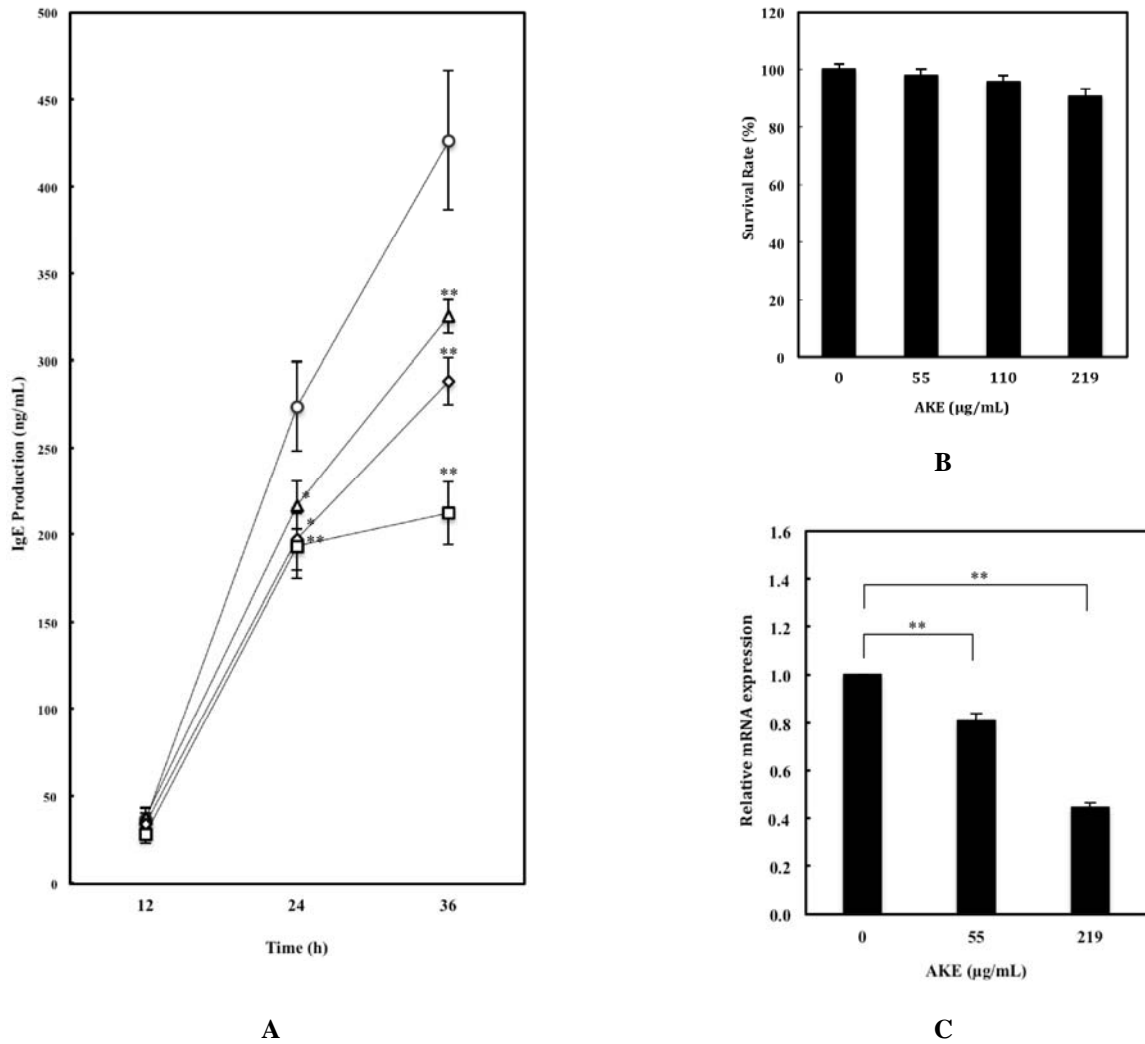


Figure 1. (A) Effect of various concentrations of AKE, 0 (circle), 55 (triangle), 110 (diamond), and 219 (square) $\mu\text{g/mL}$, on IgE production by aDNP-Hy cells in 12, 24, and 36 h incubation periods. (B) Survival rate of hybridoma aDNP-Hy cells following exposure of AKE for 24 h. (C) Relative mRNA expression in aDNP-Hy cells after 30 hours of AKE exposure. Scale bars indicate mean \pm SD. * $p < 0.05$ and ** $p < 0.01$ compared to control. Abbreviation: AKE, *asam kandis* extract

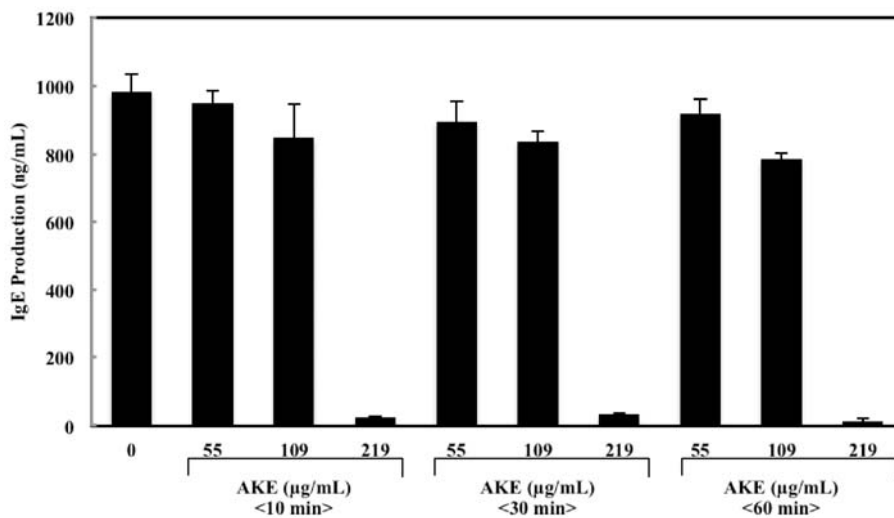


Figure 2. Effect of various concentrations of AKE, which have been previously treated with heat for 10, 30, and 60 min, on IgE production by aDNP-Hy cells in 36 h. Scale bars indicate mean \pm SD. Abbreviation: AKE, *asam kandis* extract. Scale bars indicate mean \pm SD. Abbreviation: AKE, *asam kandis* extract

In this study, we demonstrated that AKE exhibits IgE production-suppressing activity by aDNP-Hy cells. Our study showed that AKE significantly decreased IgE production by aDNP-Hy in both 24 and 36 h incubation periods in a concentration-dependent manner without any cytotoxicity. AKE also significantly decrease IgE mRNA expression. Heat treatment of AKE caused the loss of IgE production-suppressing activity, which at the same time indicating that the bioactive substances are heat labile. Moreover, heat-treated AKE showed cytotoxic effect on the cells in highest concentration that are not cytotoxic in untreated AKE. Based on our findings, we suggest that further studies are needed to determine the exact bioactive compounds of AKE that are responsible for the IgE production-suppressing activity and also evaluate the substances that are responsible for the increase of cytotoxicity of heat-treated AKE.

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