

Soybean Extract Suppresses B Cell Activation Through TLR3/TLR4 in High Fat-High Fructose Diet Mice

Soya Fasülyesi Özütü, Yüksek Oranda Yağ ve Fruktoz ile Beslenen Farelerde TLR/TLR4 Yolu ile B Hücrelerinin Uyarımını Baskılar

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Abstract

Introduction: The relationship between B cells and Toll-like Receptors (TLRs) under high fat-high fructose diet (HFFD) is still poorly understood. Isoflavone content from soybean can be modified by using biotic and abiotic elicitors to synthesize glyceollin. Glyceollin has gained much interest in recent past year due to its beneficial effects on health. The study aimed to examine the ameliorative effect of elicited soybean extract (ESE) on B cells profile in HFFD mice.

Material and Methods: Twenty-eight female *Balb/C* mice were divided into normal diet (ND), ND + ESE 104 mg/kg, HFFD, HFFD + Simvastatin 2.8 mg/kg, ESE 78 mg/kg, 104 mg/kg, and 130 mg/kg, respectively. Mice were fed with HFFD for 24 weeks and ESE was administered orally per day at last 4 weeks. At week 24, the animals were sacrificed and the spleen was collected. B cells were labeled as B220⁺TLR3⁺, B220⁺TLR4⁺, B220⁺NFκB⁻, and B220⁺NFκB⁺ and the B cells expression were measured by flow cytometry. Molecular modeling was performed by Pyrx 0.8 and visualized in PyMol.

Results: The ESE treatment significantly decreased B220⁺TLR3⁺, B220⁺TLR4⁺, and B220⁺NFκB⁺ expression and restored B220⁺NFκB⁻ expression in HFFD mice (p<0.05). Glyceollin 1 exerted the lowest binding affinity with estimated energy was -7.0 kcal/mol at NFκB.

Conclusions: ESE administration ameliorates HFFD-induced inflammation by modulating TLR3/TLR4 activation and prevents NFκB expression of B cells in HFFD mice. ESE exerts as a promising agent in the future and provides a better understanding mechanism to treat chronic inflammation caused by HFFD.

Keywords: B cell, soybean, high fat-high fructose diet, NFκB, TLR3/TLR4

Öz

Giriş: Yüksek oranda yağ ve fruktoz ile beslenme ile B hücreleri ve Toll benzeri reseptörler (TLR) arasındaki ilişki tam olarak anlaşılamamıştır. Soya fasülyesinden elde edilen izoflavonlar, biyolojik olan ve olmayan maddeler kullanılarak glyseolin üretiminde kullanılabilir. Gliseolin, sağlığa olan olumlu etkileri nedeni ile, son yıllarda özel bir ilgi alanı olmuştur. Bu çalışmada, soya fasülyesi özütünün B hücreleri üzerine olan, yüksek yağ ve fruktoz diyeti ile beslenen farelerde etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Yirmisekiz dişi Balb/c fare, normal beslenen, soya fasülyesi özütü alan, 104 mg/kg soya fasülyesi özütünün yanı sıra 2.8 mg/kg simvastatin uygulanan, sırası ile günde 78 mg/kg, 104 mg/kg ve 130 mg/kg olmak üzere en az 4 hafta soya fasülyesi özütü verilen gruplara ayrıldı. Yirmidördüncü haftada, denekler öldürülerek dalakları elde edildi. B hücrelerinin B220⁺TLR3⁺, B220⁺TLR4⁺, B220⁺NFκB⁻ ve B220⁺NFκB⁺ alt grupları akan hücre ölçer ile saptandı. Pyrx 0.8 ile moleküler modelleme yapıldı ve bu modelleme PyMol ile görüntüldü.

Bulgular: Soya fasülyesi ekstresi, yüksek oranda yağ ve fruktoz ile beslenen farelerdeki B220⁺TLR3⁺, B220⁺TLR4⁺, B220⁺NFκB⁺ ifadelerini azalttı iken B220⁺NFκB⁻ ifadesini yükseltti (p<0.05). Gliseolinin NFκB'nin bağlanma enerjisi -7 kcl/mol ile en düşük düzeyde bulundu.

Sonuçlar: Soya fasülyesi özütü, farelerde, yüksek oranda yağ ve fruktoz ile tetiklenen yangıyı TLR3/TLR4 aktivasyonunu düzenleyerek azaltır. Özüt, gelecekte, yüksek yağ ve fruktozun yarattığı kronik yangıyı tedavi etmede kullanılabilen bir ajan olarak ümit vaatmektedir.

Anahtar Kelimeler: B hücresi, soya fasülyesi, yüksek yağ, yüksek fruktoz beslenmesi, NFκB, TLR3/TLR4

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Introduction

Nutrient excess, majorly from high fat-high fructose diet (HFFD) contribute to obesity development due to accumulation lipids on adipose tissue.^[1,2] These conditions promote

a significant recruitment and release a massive amount of proinflammatory cytokines by macrophages and other immune cells in adipose tissue, which leads to chronic low-grade inflammation.^[3,4] Recent evidence suggests that low-grade inflammation may act as a pathogenic factor link to obesity-associated metabolic disorder, such as insulin resistance^[5] and type 2 diabetes mellitus.^[6]

B cells are the main parts of adaptive immunity which not only responsible to produce antibody, but also act as antigen-presenting cells in response to the microbial stimuli via TLRs.^[7–9] TLRs are one of pattern recognition receptors (PRRs) which play a pivotal role in sensing pathogen and activate various inflammatory pathway.^[10] Recently, among TLRs families, TLR3 and TLR4 were found to be involved in the progression of metabolic disorder during the high-fat diet (HFD)^[11,12] or high fructose diet.^[13,14]

In general, both TLR3 and TLR4 are active through toll-interleukine-1 receptor domain-containing adapter inducing interferon- β (TRIF)-dependent signaling pathways, whereas TLR4 is active through myeloid differentiation factor 88 (MyD88)-dependent signaling pathways.^[15] Both myd88-dependent signaling pathways and TRIF-dependent signaling pathway are essential in the early and late phase of NF κ B activation consecutively for maximum expression of cytokines.^[15,16] NF κ B activation enhances the gene expression of proinflammatory cytokine leading to inflammatory response.^[17] The overexpression of NF κ B is triggered by specific ligand TLR4, including free fatty acids (FFAs), fructose, and modified low-density lipoproteins (LDLs) and TLR3 ligand, including mRNA from damaged cells which are elevated under HFFD condition.^[14,18,19]

NF κ B is essential for B cells proliferation and differentiation.^[20] During HFD, B cells produce proinflammatory cytokine, alter T cell ratio skewed to proinflammatory phenotype, and promote hypertrophic and hyperplastic in adipose tissue.^[21,22] B cells also secrete pathogenic IgG causing impaired glucose and insulin sensitivity, which in turn worsen the inflammation.^[23] However, the role of TLR3 and TLR4 signaling pathway in B cells under HFFD condition remains poorly understood. Thus, suppressing B cell activation via TLRs is one of potential strategies to ameliorate inflammation under HFFD condition.

Soybean (*Glycine max*) is a popular plant in Leguminosae family which are globally consumed. It has the beneficial effect on health through isoflavone-rich content, including daidzein and genistein.^[24,25] Glyceollin is a phytoalexin class compound which derived from daidzein.^[26] It synthesizes to respond to various elicitors such as fungi^[26] and light.^[27] Furthermore, glyceollin synthesis could be enhanced by using the combination of fungi and light.^[27] Glyceollin has been reported to be able to exhibit antioxidant^[28], antifungal^[29], and anti-inflammatory properties.^[30,31] However, the molecular mechanism of glyceollin in B cells activation under HFFD is still not known.

This study was undertaken to understand the mechanism of isoflavone and glyceollin for suppressing NF κ B through TLR3 and TLR4 of B cells under HFFD. In addition, we applied molecular modeling to gain further insight into the possibility of isoflavone and glyceollin regulating NF κ B as a critical factor of inflammation.

Material and Methods

Reagents

Simvastatin (10 mg) was purchased from DexaMedica, Tangerang, Indonesia. Antibodies FITC-conjugated rat anti-mouse B220 (clone: RA3-6B2), PE-conjugated rat anti-mouse TLR3 (clone: 11F8), and PE/Cy7-conjugated rat anti-mouse TLR4 (clone: SA15-21) were purchased from BioLegend (San Diego, CA). PE/Cy5-conjugated rabbit anti-mouse NF κ B (Polyclonal) was purchased from Bioss Inc (Massachusetts, USA).

Soybean preparation

Soybean seeds *Anjasmoro* were purchased from Indonesian Legumes and Tuber Crop Research Institute (ILETRI), Malang District, East Java, Indonesia. Briefly, soybean seeds (100 g) were immersed in 500 mL of 70% alcohol then washed with sterile distilled water.^[32] The sterilized soybeans seed were soaked in sterilized distilled water and placed in a dark room for 24 h. Soybeans were moved into a sterile box then planted and inoculated with 1×10^7 *Saccharomyces cerevisiae* (100 g/7.5 mL). They were stored at room temperature and exposed with bulb lamp from 05.00 pm – 09.00 am for 3 consecutive days according to the previous study by Aisyah et al. with slight modification.^[27]

Soybean extraction

Soybeans were harvested after 3 days then washed three times with sterilized distilled water. After that, they were crushed and extracted with 80% ethanol (100 g/300 mL) then placed in a waterbath at 50°C in 1 h, and stirred occasionally. After that, soybeans were cooled at room temperature, then centrifuged at 14.000 rpm for 10 minutes.^[12] The extract was then filtered through 0.45 µm sterile filter (BD Falcon, NJ, USA). The filtrate was then concentrated by using rotary evaporator and then freeze-dried. The crude extract was then in storage at -20°C prior to use.

Experimental design and treatment

Three-weeks old (n=28) female *Balb/C* mice (*Mus musculus*) were purchased from The Integrated Research and Testing Laboratory-Unit IV, Gadjah Mada University. They were placed individually in a plastic cage at free pathogen chamber. The chambers were controlled at 23–25°C, 50–60% humidity, and 12 h dark/light cycle. Mice were then divided into two major group, normal diet (ND) and high fat-high fructose diet (HFFD). ND consisted of 67.27% carbohydrate, 12.73% protein, and 5.33% fat, while HFFD was based on beef tallow consisting of 53.46% carbohydrate, 8.57% protein, and 21.06% fat and the drinking water was added with 10% fructose. ND and HFFD were given for 24 weeks and the treatment was begun at 21st weeks. Previously, ND mice were divided into two groups: ND and ND + ESE 104 mg/kg. HFFD were divided into five groups: HFFD, HFFD + simvastatin 2.8 mg/kg, ESE 78, 104, and 130 mg/kg respectively. Simvastatin and ESE were given orally per day for 4 weeks. Animal welfare and experimental procedures were approved by Institutional Ethics Committee Brawijaya University (Reg. No. 647-KEP-UB).

Splenocyte isolation and staining

At the end of experiment, mice were fasted overnight and sacrificed by cervical dislocation. The spleen was collected and washed twice by using sterile PBS. It was placed into a tissue culture dish and crushed apart into a single cell suspension. A single cell suspension containing 2–3 × 10⁶ cells was then centrifuged at 2500 rpm for 5 minutes at 10°C.^[33] The supernatant was discarded and pellet was stained with antibodies against cell-surface markers, FITC-*conjugated rat anti-mouse* B220 (clone: RA3-6B2) then incubated in dark for 30 minutes at 4°C. Splenocytes had been stained previously, fixed and permeabilized

by using a cytofix/cytoperm kit (BD-Biosciences, Pharmingen) according to the manufacturer's protocol. The supernatant was discarded and pellet was stained with intracellular staining of PE-*conjugated rat anti-mouse* TLR3 (clone: 11F8), PE/Cy7-*conjugated rat anti-mouse* TLR4 (clone: SA15-21), and PE/Cy5-*conjugated rabbit anti-mouse* NFκB (Polyclonal) then incubated in dark for 30 minutes at 4°C. The staining combination used was B220/TLR3, B220/TLR4, and B220/NFκB. The final suspension was then analyzed by using Flow Cytometry (BD Cell Quest program, San Jose, CA).

Docking simulation of selected ligands

The selected compound from ESE extract including daidzein (CID: 5281708), genistein (CID: 5280961), glyceollin I (CID: 162807), glyceollin II (CID: 181883), glyceollin III (CID: 11954193), and simvastatin (CID: 54454) was downloaded from PubChem. Mouse NFκB (1NFK) was downloaded from Protein Data Bank (PDB). Firstly, all water molecule of NFκB were removed in PyMol (Schrödinger Inc., LLC) and the ligand energy was minimized by Open Babel in PyRx 0.8 (The Scripps Research Institute, California) before docking simulation. Autogrid for ligand docking to NFκB was prepared at the same center and size of the box at 30 Å and coordinates were set on x=-1.1958 Å; y=9.0149 Å; z=19.7598 Å^[34] by AutoDock Vina in Pyrx 0.8. Docking visualization was then performed by PyMol.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The relative number (%) of B220⁺TLR3⁺, B220⁺TLR4⁺, B220⁺NFκB⁻, and B220⁺NFκB⁺ were analyzed by one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) to calculate the statistical significance between groups. *p-value* < 0.05 was considered significant. Statistical analysis was performed by Microsoft Excel for Windows.

Results

The elicited soybean extract prevented B cell activation through TLR3 and TLR4

Fig. 1a revealed that B220⁺TLR3⁺ expression of the HFFD groups was significantly higher compared to ND groups (*p*<0.05). Interestingly, there were no significant differences found in B220⁺TLR3⁺ expression between ESE dose 78, 104 and 130 mg/kg and normal. In addition, there was no significant change in ND + ESE 104 mg/kg

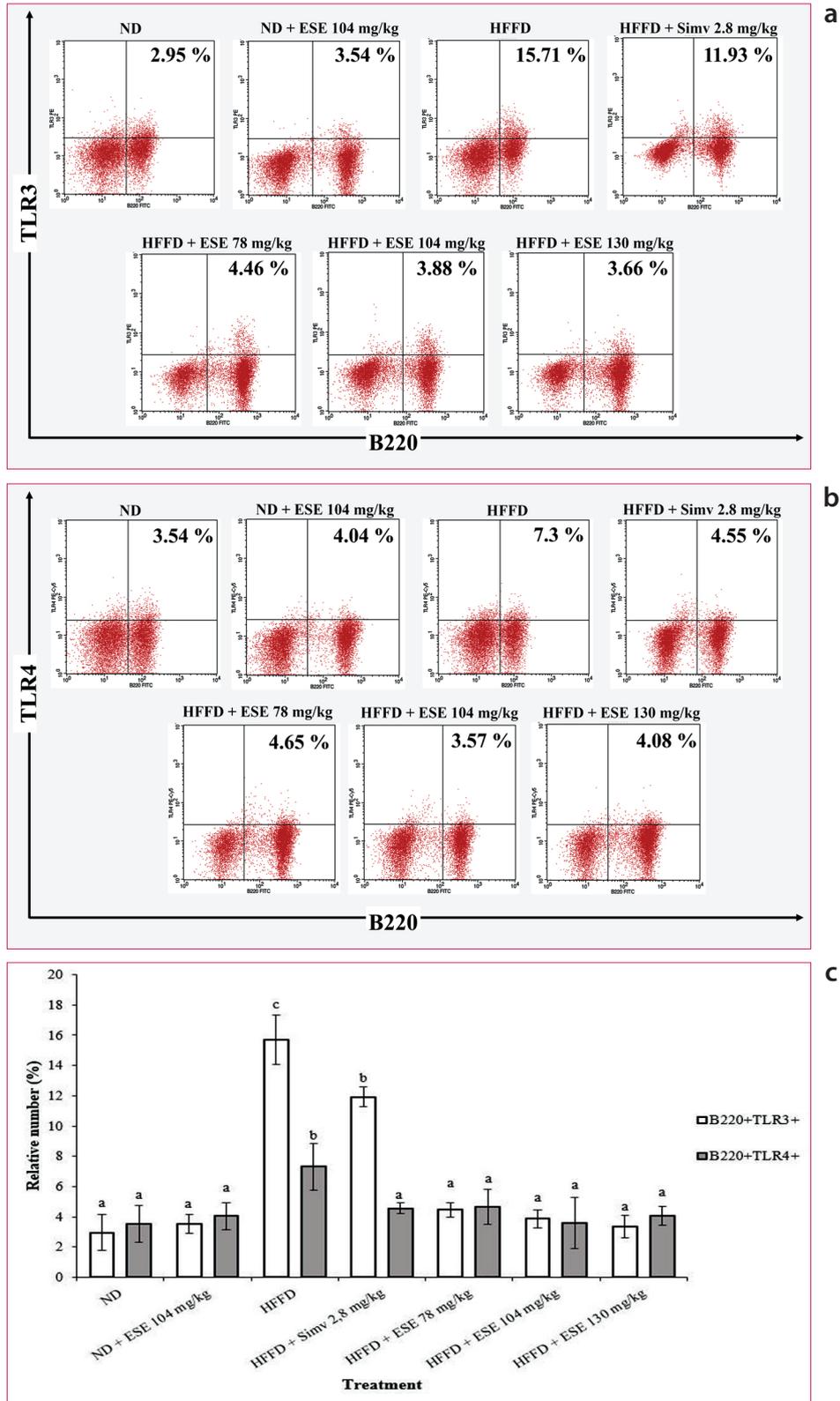


Figure 1. Flow cytometry analysis exhibited B cells expression. The value upright indicated the proportion of (a) B220⁺TLR3⁺ on splenocytes, (b) B220⁺TLR4⁺ on splenocytes and (c) B220⁺TLR3⁺ and B220⁺TLR4⁺ expressions were represented as mean ± SD (n=4 for each groups). The different letter on the chart was considered significantly different for each group at p<0.05 and vice versa based on DMRT pos hoc test. ND=Normal Diet; ESE=Elicited Soybean Extract; HFFD=High Fat-Fructose Diet.

compared to ND only. Surprisingly, treatment with ESE had affected more on B220⁺TLR3⁺ expression compared to mice administered Simvastatin at a dose of 2.8 mg/kg BW (Fig. 1c).

B220⁺TLR4⁺ expression of the HFFD groups was significantly higher compared to ND groups (Fig 1b) ($p < 0.05$). ESE treatment given under HFFD reduced B220⁺TLR4⁺ expression compared to HFFD only. Interestingly, ESE at doses 78, 104, and 130 mg/kg improved B220⁺TLR4⁺ expression towards near normal and were not significantly different compared to ND (Fig. 1c). Moreover, ESE at dose 104 mg/kg BW did not significantly change B220⁺TLR4⁺ expression in ND group.

The elicited soybean extract decreased NFκB expression in B cell

B220⁺NFκB⁻ expression of HFFD group decreased compared to that of ND group (Fig. 2a). The expression

of B220⁺NFκB⁻ significantly increased ($p < 0.05$) by administration of ESE 130 mg/kg. The treatment of simvastatin at a dose of 2.8 mg/kg, ESE 78, and 104 mg/kg increased B220⁺NFκB⁻ towards near normal, although it was no significant difference in ESE at doses of 78 mg/kg and 104 mg/kg compared to HFFD (Fig. 2b). Next, B220⁺NFκB⁺ expression of HFFD group had elevated when compared to ND group (Fig. 2a). By contrast, treatment with ESE at doses of 78, 104, and 130 mg/kg decreased B220⁺NFκB⁺ expressions. Interestingly, ESE at doses of 78 and 104 mg/kg increased B220⁺NFκB⁺ expressions towards near normal, although there were no significant differences found when compared to control groups ($p < 0.05$) (Fig. 2b). However, ESE at a dose of 130 mg/kg BW did not significantly change B220⁺NFκB⁻ and B220⁺NFκB⁺ expressions in ND mice. ESE at a dose of 130 mg/kg BW seemed to be the optimal dose to increase the rates of B220⁺NFκB⁻ and B220⁺NFκB⁺ cells.

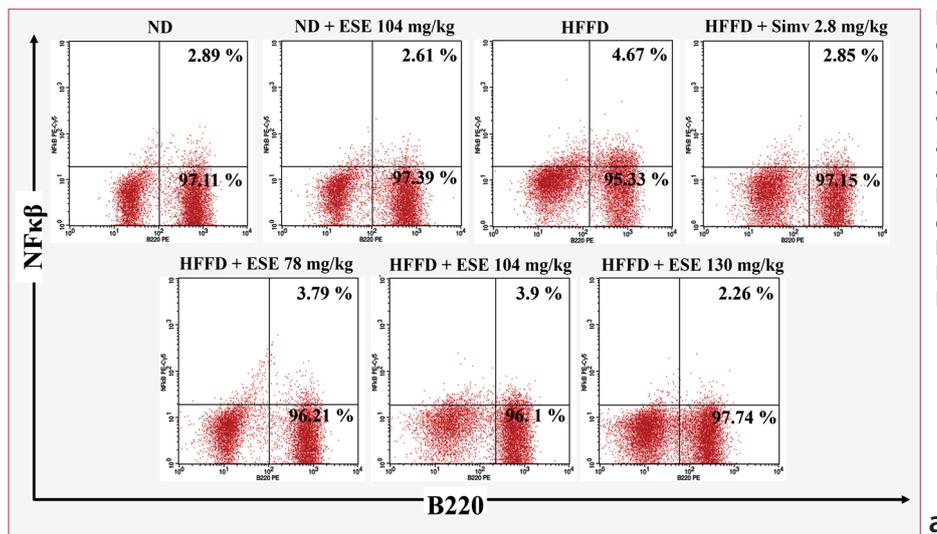
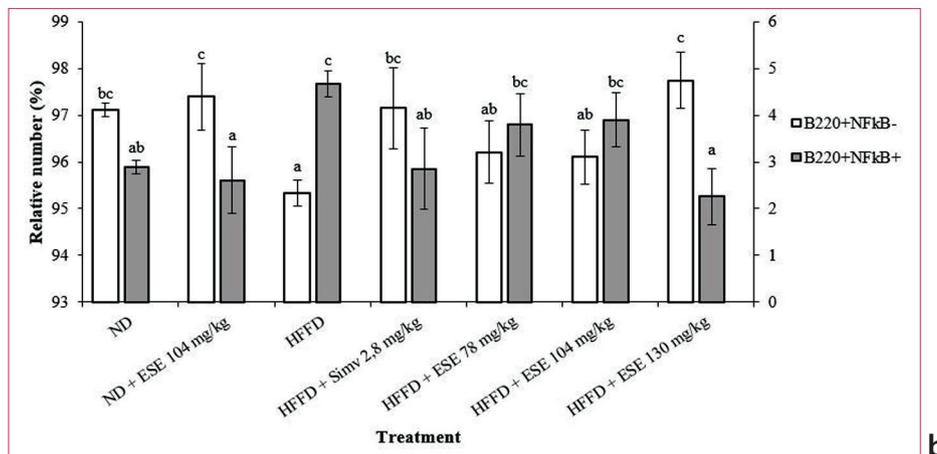


Figure 2. Flow cytometry analysis exhibited B cells expression. (a) The B220⁺NFκB⁻ proportion on splenocytes was indicated in the value upright, while the B220⁺NFκB⁺ proportion on splenocytes was indicated in the low-right, (b) B220⁺NFκB⁻ and B220⁺NFκB⁺ expressions were represented as mean ± SD (n=4 for each group). The different letter on the chart was considered significantly different for each group at $p < 0.05$ and vice versa based on DMRT pos hoc test. ND= Normal Diet; ESE = Elicited Soybean Extract; HFFD = High Fat-Fructose Diet.



b

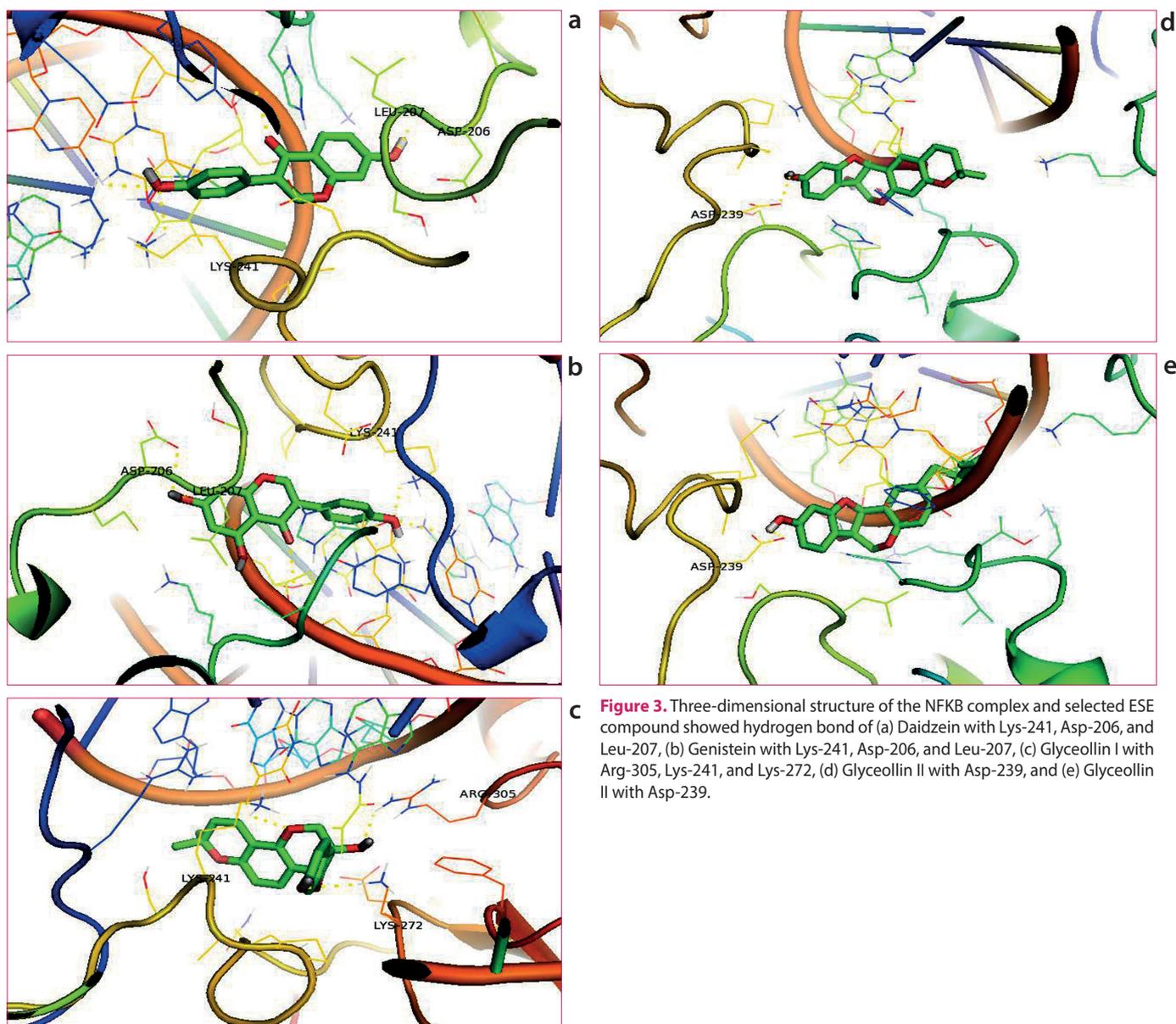


Figure 3. Three-dimensional structure of the NFκB complex and selected ESE compound showed hydrogen bond of (a) Daidzein with Lys-241, Asp-206, and Leu-207, (b) Genistein with Lys-241, Asp-206, and Leu-207, (c) Glyceollin I with Arg-305, Lys-241, and Lys-272, (d) Glyceollin II with Asp-239, and (e) Glyceollin II with Asp-239.

Molecular docking analysis for the selected inhibitors on NFκB

Table 1 depicted the result of docking molecules daidzein, genistein, glyceollin I, glyceollin II, glyceollin III, and simvastatin around NFκB. Glyceollin I has the lowest binding affinity with NFκB. Interestingly, glyceollin I, glyceollin II, glyceollin III, daidzein, and genistein appear to tightly bind around NFκB better than simvastatin.

Moreover, we found that specific amino acids were needed for binding between NFκB and selected ligands. Our docking result suggested daidzein and genistein form the hydrogen bond with three amino acids, including Lys-241, Asp-206, and Leu-207 of NFκB (Fig. 3a, 3b).

Table 1. The estimated of binding affinity between NFκB and selected ESE compound

Ligand	Binding Affinity (kcal/mol)
Daidzein	-6.4
Genistein	-6.4
Glyceollin I	-7.0
Glyceollin II	-6.7
Glyceollin III	-6.6
Simvastatin	-5.6

Glyceollin I formed the hydrogen bond with three amino acids, including Arg-305, Lys-241, and Lys-272 of NFκB (Fig. 3c). Glyceollin II and III formed a hydrogen bond with one amino acids, Asp-239 of NFκB (Fig. 3d, 3e).

Discussion

Nowadays, obesity has gained much attention due to its association with health problems linked to the decreased life quality and increasing health care cost.^[2,35] In recent years, there is much evidence demonstrated the beneficial effect of isoflavone and glyceollin due to their derivative in the metabolic disorder.^[36,37] Our previous study has proved that oral administration of ESE for 4 weeks significantly restored regulatory T cells (Tregs) in HFFD mice.^[38] However, the underlying mechanism of ESE which involves the central role of B cells remains unclear. In the present study, for the first time, we reported the role of ESE towards B cell population under HFFD.

The TLRs function is closely related to dendritic cells or macrophages which acts as the first line defense. TLRs have a primary role in recognizing PAMPs or DAMPs which in turn generate excessive proinflammatory cytokine.^[17] In B cells, TLRs have a direct effect on their activation and antibody production. They also support B cells receptor (BCR) to detect the microbial origin which in turn provide the optimal antibodies response.^[7] A recent study reported that B cells become more active to produce cytokine, chemokines, and antibodies under hyperlipidemic condition through TLRs signaling pathway.^[39] Moreover, our result demonstrated that an HFFD trigger B cells activation through TLR3/TLR4 signaling pathway. In contrast, the ESE treatment reduced B220⁺TLR3⁺ and B220⁺TLR4⁺ expression.

Receptor dimerization is a critical step to activate downstream cascade signaling pathways of TLRs.^[15] Genistein was reported to prevent LPS binding to TLR4 which in turn delayed the further inflammatory signal.^[40] Daidzein, another soy isoflavones, was reported to inhibit TLR4/MyD88 signaling pathway.^[41] Glyceollin is a class of phytoalexin which is synthesized by soybean under stress condition.^[26] Resveratrol, a phytoalexin contained in grape was reported to inhibit TLRs through TRIF-dependent signaling pathway.^[42] In our study, glyceollin contained in ESE may participate to disrupt ligand binding to TLR3/TLR4, but the exact mechanism remains to be further determined.

Our B220⁺TLR3⁺ and B220⁺TLR4⁺ results were consistent with B220⁺NFKB⁻ expression. The decrease of B220⁺NFKB⁻ expression followed the decrease of B220⁺TLR3⁺ and B220⁺TLR4⁺ expression. NFKB is known as a master key of the transcription factor which is closely

linked to inflammation and it plays an important role in the progression of metabolic disorder.^[43] NFKB activation mediated by TLRs assists an expression of Blimp-1, a transcription factor which is responsible for B cells differentiation.^[44] Daidzein and genistein were reported to avoid p65 NFKB, and IκB-α phosphorylation when leading to down-regulation of NFKB activation.^[40,41] On the other hand, glyceollin declines phosphorylation of IKKα/β, an upstream kinase of IκB in NFKB which in turn prevents NFKB translocation to bind with DNA in the nucleus.^[31] Furthermore, NFKB inhibition may suppress IgG1 and IgE synthesis^[45], while IgG is found to increase under HFD condition.^[23] Our result suggested that inhibitory effect of ESE on TLR3/TLR4 signaling pathway contributes to reducing B cells activation under HFFD condition.

To further elucidate the mechanism of glyceollin intervention, we performed molecular docking to verify the role of selected ESE compound at NFKB. Our study demonstrated glyceollin I has the lowest binding affinity to NFKB followed by glyceollin II, glyceollin III, daidzein, genistein, and simvastatin respectively. Glyceollin I interacted with Arg-305 and Lys-272 known as dimerization region at p50 NFKB.^[34] Next, glyceollin I forms a hydrogen bond with Lys-241. In addition, daidzein and genistein interacted with Lys-241. Lys-241 is known as primary residue which interacts with consensus DNA sequences of κB site resulting the NFKB failed to bind DNA Binding Region (DBR).^[46] Furthermore, daidzein and genistein also form a hydrogen bond with Asp-206, Leu-207, and Asp-239 which have a pivotal role in binding NFKB at DBR.^[46] These results suggest that glyceollin I, glyceollin II, glyceollin III, daidzein, and genistein may have different role to prevent NFKB activation, which in turn alleviate the inflammation.

In conclusion, ESE had ameliorative effect under HFFD condition related to inflammation. Our result demonstrated that ESE do not significantly change B cells expression when given in ND. Moreover, we suggest the underlying mechanism of ESE overcoming inflammation is through preventing the ligand binding for TLR3/TLR4. Therefore, ESE may interrupt the binding of NFKB at DBR which in turn inhibits B cells differentiation into the plasma cell and diminishes overexpression of pathogenic antibodies and cytokine proinflammatory. Our finding exhibits ESE as a promising alternative treatment in the future for obesity-associated inflammation induced by HFFD.

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