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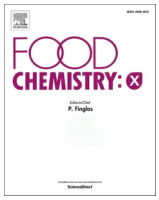

Structural, physicochemical, and immune-enhancing properties of edible insect protein isolates from *Protaetia brevitarsis* larvae

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ABSTRACT

Edible insects are promising future food resources globally. Herein, the structural, physicochemical, and biofunctional properties of edible insect protein isolates (EPIs) extracted from *Protaetia brevitarsis* larvae were investigated. The results showed that EPIs have a high total essential amino acid content; moreover, β-sheet is the major secondary protein structure. The *EPI* protein solution was highly soluble and electrically stable and did not aggregate easily. In addition, EPIs exhibited immune-enhancing properties; *EPI* treatment of macrophages induced the activation of macrophages and consequently promoted the production of pro-inflammatory mediators (NO, TNF-α, and IL-1β). Moreover, macrophage activation of EPIs was confirmed to occur through the MAPK and NF-κB pathways. In conclusion, our results suggest that the isolated *P. brevitarsis* protein can be fully utilized as a functional food material and alternative protein source in the future food industry.

1. Introduction

The United Nations has announced that by 2050 the world population will reach 9.7 billion (Borges, da Costa, Trombete, & Câmara, 2022). Thus, sustainable development requires solutions to cope with the exploding global population. As the population increases, food security has become a global concern, with studies being actively conducted to solve issues regarding this topic (Dickie, Miyamoto, & Collins, 2019; Cruz-López et al., 2022). Traditional food sources are not sufficient to meet the needs of the growing global population. Thus, research on new, more efficient, sustainable, and nutritionally superior alternative food sources is highly needed (Mariutti et al., 2021). Research on edible insects is attracting considerable attention (Kim, et al., 2021; Lee, Cha, Kim, Choi, & Jang, 2021) owing to their several advantages as alternative protein sources over conventional protein sources (Lee et al., 2023). Edible insects reduce ammonia generation, have good feed conversion ratios, and reduce the amount of water required for breeding animal sources (Lee et al., 2021a). In addition, it is expected that they will play an important role in achieving the global environmental goal of carbon neutrality because of their significantly lower CO_2 production, a representative greenhouse gas, compared to that of the existing livestock industry (Vauterin, Steiner, Sillman, & Kahiluoto, 2021).

Protaetia brevitarsis (PB, white-spotted flower chafer) is a representative edible insect (Lee et al., 2021a) containing 58% protein as the major component, 17% lipid, 5% fiber, and 8% ash (Noh et al., 2018). PB is attracting attention as an alternative protein source owing to its high protein and essential amino acid contents (Kim et al., 2021). Lee, Jo, Yong, Choi, and Jung (2021) reported that PB larvae protein showed higher digestibility compared to beef loin protein; therefore, they suggested that PB larvae protein could be used as an alternative protein source for the elderly (Ham et al., 2021). Moreover, Kim et al. (2021) studied the changes in protein properties according to the organic solvent used for defatting PB larvae protein; changes in the functional properties (emulsion capacity, emulsion stability, foam capacity, and foam stability) of proteins according to solvent treatment were determined to verify the applicability of PB larvae as a protein source. In addition, in a previous study, defatting PB larvae protein reduced its off-flavor and increased its pleasant flavor (Lee et al., 2021a), therefore reducing consumers' prejudice against edible insects. Such a feature is important for increasing their prospect as an alternative protein source. The combination of research on the physiological activity along with research on the biological functional activity of insect protein is expected to afford new possibilities for commercial use (Kim et al., 2022b). In addition to the use of PB protein as an alternative protein source in the

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form of powder, the possibility of using it as a functional food material can be considered.

The immune response system is classified into innate and adaptive immune systems (Ren, Zhang, & Zhang, 2021). To prevent various diseases caused by pathogenic bacteria, a normal immune response is essential (Cai et al., 2021). However, when people age or fall ill, their immune function decreases and a normal immune response cannot be obtained, rendering them vulnerable to external pathogens (Lee et al., 2017). Therefore, studies on materials that enhance the immune response are being actively conducted to develop immune-enhancing materials that can improve the human defense system (Jiang et al., 2021a). Among the many immune cells, macrophages play a key role at the forefront of the immune system (Geng, Hu, Liu, Wang, & Zhang, 2018). They are activated when an external stimulus is recognized; activated macrophages produce immune mediators essential for immune response, such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), and pro- or anti-inflammatory cytokines (e.g., tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6) (Lee, Lee, & Paik, 2022). In addition, macrophages involved in immune mediator production are mainly activated through the activation of several cell signaling pathways such as the nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) (Geum et al., 2020). If the immune system is weakened and the macrophage function is not activated, the host's homeostasis can be fatally damaged (Kim, Lee, Yeo, Hwang, & Park, 2022). Research on natural substances that can activate macrophages is currently underway (Geum et al., 2020; Jiang, et al., 2021a; Kim et al., 2022a; Lee et al., 2022).

Herein, edible insect protein isolates (EPIs) were extracted from PB and their structural, physicochemical, and bio-functional properties were studied simultaneously. Thus far, few studies have investigated these properties simultaneously. The prospects of the use of EPIs in food were investigated by studying their structural and physicochemical properties together. Meanwhile, the bio-functional properties of EPIs, particularly the ability to activate the immune system, which is essential for immune-deficient patients and the elderly, were investigated to confirm the potential application of EPIs as a health functional food material.

Hypothesis: Edible insect protein isolates from *Protaetia brevitarsis* larvae can be utilized not only as a potential food source but also as an immune-enhancing functional food material for immune-deficient patients and the elderly.

2. Materials and methods

2.1. Materials

Protaetia brevitarsis larvae (age: 3rd instar, form: freeze-dried powder) were obtained from FARMBANG (Sunchang, Korea). For cell culture, Dulbecco's Modified Eagle's Medium (DMEM), antibiotics, fetal bovine serum (FBS), and phosphate buffered saline (PBS) were purchased from Gibco (Grand Island, NY, USA). Griess reagent, lipopolysaccharide (LPS), amino acids standards, and protease/phosphatase inhibitor cocktail were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). For cytokine quantification, TNF-α and IL-1β ELISA kits were obtained from Abcam Inc. (Cambridge, UK). For western blot analysis, the primary (iNOS, p-p38, p38, p-ERK, ERK, p-JNK, JNK, p-p65, p65, p-IκB-α, IκB-α, and β-actin) and secondary antibodies (HRP-conjugated antibody) were obtained from Cell Signaling Technology (Danvers, MA, USA). Other reagents for western blot analysis, including EveryBlot Blocking Buffer, RIPA Buffer, Laemmli Sample Buffer, Tris-buffered saline with 0.5% Tween-20 (TBST), and enhanced chemiluminescence (ECL, Clarity Western ECL substrate) reagent were purchased from Bio-Rad (Hercules, CA, USA). All solvents used in this study were of analytical grade.

2.2. Preparation of edible insect protein isolate

Before the preparation of EPIs from PB, the PB powder was defatted using *n*-hexane according to a previously reported method (Kim et al., 2021). Briefly, PB powder (200 g) was dissolved in *n*-hexane (1 L) by stirring for 1 h. The supernatant was then discarded and fresh *n*-hexane (1 L) was added, followed by stirring for 1 h. This process was repeated five times. Then, after the supernatant was removed, the remaining *n*-hexane was evaporated overnight under a fume hood.

The defatted PB powder was extracted using distilled water (DW) and saline solution (0.58 M, pH 8.3); 200 g of PB powder was mixed with 1 L of DW or saline solution and incubated overnight at 4 ℃. After incubation, centrifugation (12,000 g, 30 min) was performed, and the resulting supernatant was freeze-dried. The sample obtained using DW was named *EPI*-W, whereas the sample obtained using the saline solution was named *EPI*-S.

2.3. Structural properties of edible insect protein isolate

2.3.1. Amino acid analysis

First, the EPIs solution was hydrolyzed using 6 M HCl under nitrogen (105 ℃, 24 h). The hydrolysate was vacuum-dried at 40 ℃ and redissolved in 0.02 M HCl. After filtration using a membrane filter (0.20 μm), the filtered sample was analyzed for its amino acid content using an amino acid analyzer equipped with an ion exchange resin column (L-8800; Hitachi, Tokyo, Japan).

2.3.2. SDS-PAGE

The protein molecular weight distribution of EPIs was determined using the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Lee et al., 2021a). EPIs were mixed with the sample buffer at a ratio of 1:4 (v/v). The mixture was heated, cooled, and separated using a 12% SDS-PAGE gel. After staining the gel with staining buffer, the molecular weight distribution was confirmed using the used protein molecular weight standard marker (Bio-Rad).

2.3.3. Determination of the protein secondary structure

The secondary structures of the EPIs were estimated using circular dichroism (CD) spectroscopy (Chirascan, Applied Photophysics Ltd., Surrey, UK). The protein concentration was regulated at 0.2 mg/mL, and the optical path length of the cells was set to 1 mm. A scan rate of 100 nm/min was used to measure the molecular ellipticity, and the scan range was set to 190–260 nm. The CEVA Deep Neural Network software was used to estimate the contents of the secondary structures.

2.4. Physicochemical properties of edible insect protein isolate

2.4.1. Particle size, polydispersity index (PDI), and zeta potential determination

To measure the mean particle size, PDI, and zeta potential of the *EPI* solution, DLS analysis was performed using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK) at a detection angle, wavelength, and temperature of 90°, 632 nm, and 25 ℃, respectively. Prior to the analysis, EPIs were diluted 10-fold with DW to prevent non-specific scattering, and all samples were measured in triplicate.

2.5. Immune-enhancing properties of edible insect protein isolate

2.5.1. Cell culture and cell viability

The RAW 264.7 macrophages (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 10% FBS and 1% antibiotics at 37 ℃ under 5% CO_2.

To confirm the effect of EPIs on cell viability, the WST cell proliferation kit (EZ-Cytox, DoGen-Bio, Seoul, Korea) was used. Briefly, RAW 264.7 macrophages were seeded in a 96-well plate. After incubation for 4 h, various concentrations of EPIs (3.13–100 μg/mL) were treated in

each well for 24 h. Thereafter, the WST solution was treated in each well and incubated for 1 h. The absorbance was measured using a microplate reader (Molecular Devices, San Jose, CA, USA) at 450 nm.

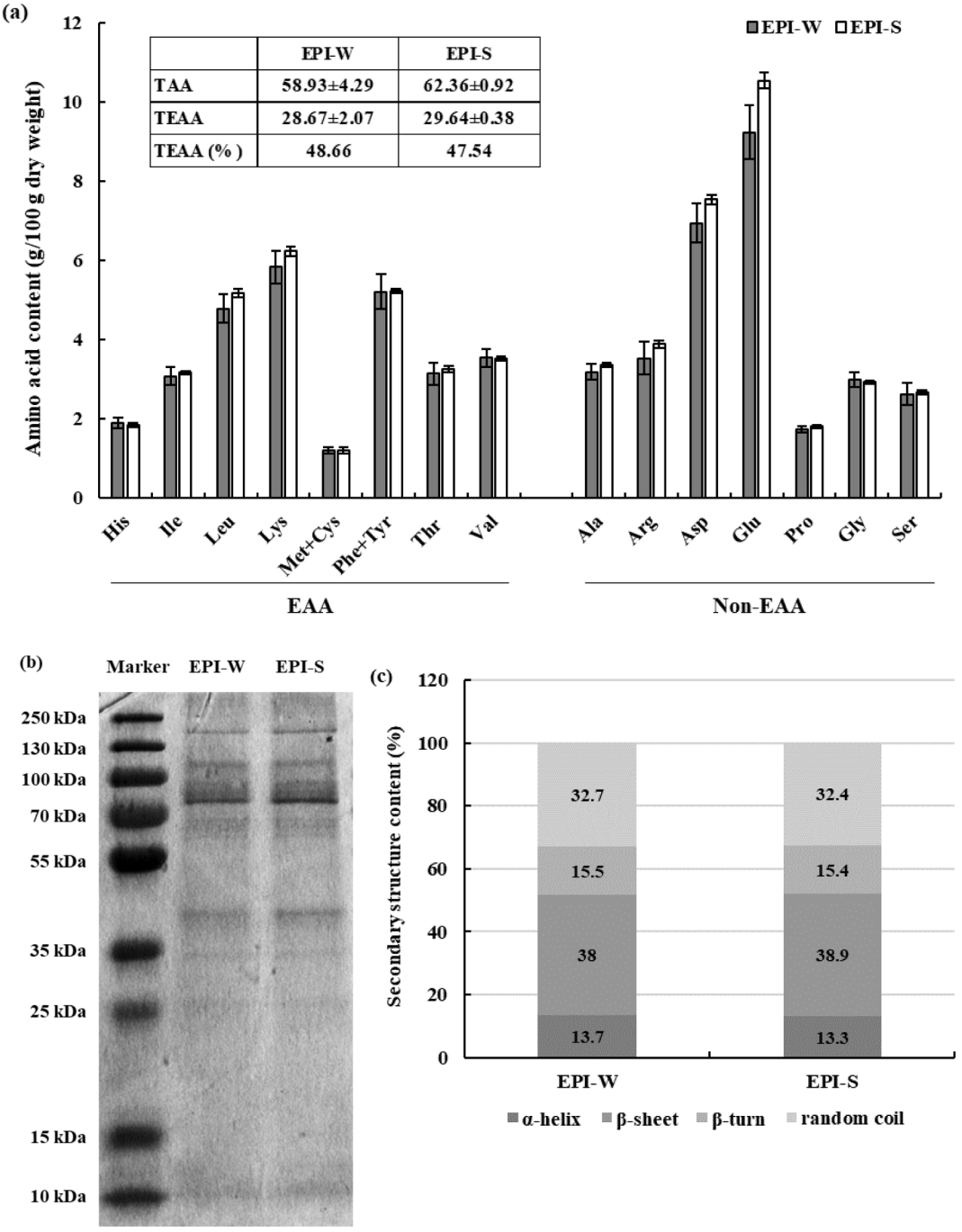
2.5.2. Effects on the production of NO and pro-inflammatory cytokines (TNF- α and IL-1 β)

The effects of EPIs on the NO production in RAW 264.7 macrophages were determined using the Griess reagent (Lee et al., 2022). RAW 264.7 macrophages were seeded in a 96-well plate. After incubation for 4 h, various concentrations of EPIs (3.13–100 μg/mL) were treated in each well for 24 h. Thereafter, the culture supernatant (100 μL) was mixed with an equal volume of Griess reagent. After incubation for 15 min, the absorbance was measured at 540 nm using a microplate reader.

The effects of EPIs on the TNF-α and IL-1β production were determined using an ELISA kit. RAW 264.7 macrophages were seeded in a 24-well plate. After incubation for 24 h, various concentrations of EPIs (10 and 100 μg/mL) were treated in each well for 24 h. The TNF-α and IL-1β levels in the wells were determined using an ELISA kit following the manufacturer's instructions.

2.5.3. Western blot analysis

The effects of EPIs on the expression of protein (iNOS, MAPK pathway protein, and NF-κB pathway protein) were determined using western blot analysis. RAW 264.7 macrophages were seeded in each cell culture dish (60 mm) and incubated for 24 h. EPIs (10 and 100 μg/mL) were then treated in each dish. The total protein was extracted using the



	EPI-W	EPI-S
TAA	58.93±4.29	62.36±0.92
TEAA	28.67±2.07	29.64±0.38
TEAA (%)	48.66	47.54

Fig. 1. Structural properties of EPIs: amino acid profile (a), SDS-PAGE (b), and secondary structure content (c). Values are expressed as the mean ± standard deviation. EAA, essential amino acids; non-EAA, non-essential amino acids; TAA, total amino acids; TEAA, total essential amino acids.

RIPA buffer (with protease/phosphatase inhibitors) after 30 min (for detecting proteins related to MAPK and NF-κB pathways) or 24 h (for detecting iNOS). The extracted proteins were quantified using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The same concentration of protein was separated by SDS-PAGE (12% gel) and then transferred to a PVDF membrane (Bio-Rad). After blocking with blocking buffer for 30 min, the PVDF membrane was incubated with the target primary antibody overnight at 4 ℃. The membrane was then washed using TBST for 20 min and incubated with the secondary antibody at room temperature for 2 h. After incubation, the membrane was washed with TBST for 40 min. The proteins were detected using the ECL reagent and observed with ChemiDoc™ XRS+ (with Image Lab™ Software, Bio-Rad).

2.5.4. Blocking assay

To confirm which cell signaling pathways were involved in the activation of RAW 264.7 macrophages by *EPI* treatment, a blocking assay using a specific inhibitor (SB202190, p38; SP600125, JNK; PD98059, ERK; and PDTC, NF-κB) was performed (Lee et al., 2022). Briefly, RAW 264.7 macrophages were seeded in a 24-well plate. After 24-h incubation, the specific inhibitor (10 μM) and EPIs (100 μg/mL) were co-treated in each well for 24 h. Finally, the amount of produced NO was determined using the Griess reagent.

2.6. Statistical analysis

The data are presented as means ± standard deviation ($n = 3$). Statistical analysis was conducted using SPSS (ver. 20; SPSS Inc., Chicago, IL, USA). A student's *t*-test or one-way analysis of variance using Duncan's multiple comparison tests was performed ($P < 0.05$).

3. Results and discussion

3.1. Structural properties of EPIs

3.1.1. Amino acid composition of EPIs

The amino acid composition of *EPI*-W and *EPI*-S is shown in Fig. 1a. The total amino acid contents of *EPI*-W and *EPI*-S were 58.93 and 62.36 g/100 g dry weight, respectively. The amino acid analysis of *EPI*-W and *EPI*-S confirmed that Leu, Lys, and Phe + Tyr were the major essential amino acids, while Asp and Glu were the major non-essential amino acids. The content ratios (%) of essential amino acids to total amino acids of *EPI*-W and *EPI*-S were 48.66% and 47.54%, respectively. These ratios were superior to those of proteins extracted from other insects (*Antheraea pernyi* pupae, 45.10%; *Bombyx mori* pupae, 43.44%; and *Tenebrio molitor* larvae, 40.20%) reported in previous studies (Rao, 1994; Ravzanaadii, Kim, Choi, Hong, & Kim, 2012; Zhou & Han, 2006).

3.1.2. SDS-PAGE

The SDS-PAGE profiles of *EPI*-W and *EPI*-S are shown in Fig. 1b. According to Lee et al., the defatting *P. brevitarsis* protein electrophoresis exhibited a main band around 75 kDa and several dark bands at approximately 150, 37, and 10 kDa (Lee et al., 2021a). In this study, the *EPI*-W and *EPI*-S main bands appeared at approximately 75 kDa and the protein bands appeared at approximately 250–100, 55–35, and 10 kDa, which is in agreement with previous studies. However, the SDS-PAGE analysis exhibited no difference in the protein pattern depending on the solvent used for protein extraction.

3.1.3. Structural analysis of EPIs

The intermolecular hydrogen bond plays an important role in maintaining the secondary structure of a protein. This hydrogen bond allows the polypeptides that constitute the protein to form specific structures (α-helix, β-sheet, β-turn, and random coil) (Yu-Tong, Chun, Yue-Ming, Bao, & Xiong, 2022). The results of the secondary structure analysis of EPIs are presented in Fig. 1c. *EPI*-W showed a secondary structure composition of 13.7% α-helix, 38% β-sheet, 15.5% β-turn, and 32.7% random coil, whereas *EPI*-S showed a secondary structure composition of 13.3% α-helix, 38.9% β-sheet, 15.4% β-turn, and 32.4% random coil. As a result, it was confirmed that the major secondary structures of *EPI*-W and *EPI*-S were β-sheet and random coil.

Similar to our results, the major secondary structures of *T. molitor* larvae protein were 38.6% β-sheet and 38.6% random coil (Jiang et al., 2021c). However, proteins of silkworm pupae (42.2% α-helix, (Attaribo et al., 2020)) and black cricket (80.6% α-helix, (Santiago, Fadel, & Tavares, 2021)) showed that α-helix was a major secondary protein, and their secondary structural protein composition was slightly different from that reported here. This result implies that the types of proteins isolated from insects vary depending on the insect species. In addition, no significant difference in the composition ratio of the secondary structure according to the method of protein extraction was observed ($P > 0.05$).

3.2. Physicochemical properties of EPIs

3.2.1. Particle size, PDI, and zeta potential of EPIs

The physicochemical properties of EPIs defined by DLS analysis are shown in Table 1. In the results of mean particle size and PDI, no significant difference was observed between *EPI*-W and *EPI*-S ($P > 0.05$).

The particle sizes of *EPI*-W and *EPI*-S were measured to be 84.30 ± 0.70 and 85.78 ± 1.75 nm, respectively, and their PDI were 0.51 ± 0.01 and 0.51 ± 0.03, respectively. The correlation between protein particle size and solubility shows that the smaller the size, the higher the solubility (Pereira et al, 2016). Therefore, studies for achieving a low particle size in various protein sources are underway (Eze, Chatzifragkou, & Charalampopoulos, 2022; Zhang, Pan, Shen, Cai, Zheng, & Miao, 2018). Soy or rice protein, which is a representative protein source in the food industry, has a particle size of 400 nm, which can be reduced to 200 nm if additional processing (ultrasonication) is performed (Eze et al., 2022; Zhang et al., 2018). In this study, both *EPI*-W and *EPI*-S showed particle sizes of <100 nm, indicating that they can be used as a new protein source with high solubility. The PDI presented together with the particle size is a value indicating the homogeneity of dispersed protein particles. A high value means that the particles are non-uniform. In general, a PDI of approximately 0.5 can be considered accurate (Eze et al., 2022) and in this study, both *EPI*-W and *EPI*-S showed a PDI value of 0.51.

The zeta potential is an essential indicator of stability in protein solutions. A high zeta potential value (positive or negative) in a protein solution means that the repulsive force between the protein particles is large, indicating that they are electrically stable and do not aggregate easily (Zhang et al., 2018). High stability has a positive effect on the solubility of proteins, therefore increasing their industrial value (Yousefi & Abbasi, 2022). Here, the *EPI*-W and *EPI*-S solutions exhibited zeta potential values of −14.20 ± 1.21 and −16.77 ± 0.81 mV, respectively. Moreover, *EPI*-S was confirmed to have a significantly higher zeta potential value ($P < 0.05$) than *EPI*-W, suggesting that it can maintain a more stable protein solution than *EPI*-W. Similar studies on the zeta potential of edible insect proteins have been reported. Queiroz et al. (2021) reported that the protein solution extracted from the black soldier fly larvae (*Hermetia illucens*) has a zeta potential of −15.63 mV. According to Jiang et al. (2021c), the zeta potential of the protein of *T. molitor* larvae was –14.40 mV. These zeta-potential values are similar to the zeta-potential values of *EPI*-W and *EPI*-S proteins isolated from

Table 1
Particle size, polydispersity index (PDI), and zeta potential of EPIs.

Sample	Particle size (nm)	PDI	Zeta potential (mV)
EPI-W	84.30 ± 0.70	0.51 ± 0.01	−14.20 ± 1.21
EPI-S	85.78 ± 1.75 ^{ns}	0.51 ± 0.03 ^{ns}	−16.77 ± 0.81*

ns: not significant difference between samples in same column ($P > 0.05$).
* mean statistical difference between samples in same column ($P < 0.05$).

P. brevitarsis larvae in this study.

3.3. Immune-enhancing properties of EPIs

3.3.1. EPIs increased the production of NO and iNOS

First, the cell viability was measured using the WST cell proliferation reagent to confirm whether *EPI* treatment was toxic to RAW 264.7 macrophages. As shown in Fig. 2a, *EPI* treatment had no effect on cell viability at the tested concentrations (3.1–100 μg/mL). Cell viability was significantly lowered to 85% after treatment with LPS ($P < 0.05$), whereas no significant difference was observed with the negative control group after *EPI*-W and *EPI*-S treatment ($P > 0.05$).

Then, the effects of *EPI*-W and *EPI*-S treatment on NO production of RAW 264.7 macrophages were determined using the Griess reagent. Fig. 2b shows that treatment with *EPI*-W and *EPI*-S increased the production of NO significantly ($P < 0.05$). Treatment with 100 μg/mL of *EPI*-W and *EPI*-S, produced 17.3 ± 0.9 and 17.7 ± 1.0 μM of NO in RAW 264.7 macrophages (Fig. 2b). At high concentrations, no significant difference between *EPI*-W and *EPI*-S was observed ($P > 0.05$): however, treatment with low concentrations (6.2–25 μg/mL) *EPI*-S resulted in significantly high NO production ($P < 0.05$).

NO is produced when macrophages are activated and is an important mediator for the inflammatory response (Lee et al., 2022). It has multiple biological functions, such as inducing tumor cell death, wound healing, regulating vasodilation, and eliminating foreign microbes (Li et al., 2015). In this study, *EPI*-W and *EPI*-S treatment induced the activation of RAW 264.7 macrophages and showed the effect of increasing the production of NO, a representative pro-inflammatory molecule. Meanwhile, the production of NO is closely related to the enzymes iNOS that synthesize them. Therefore, the effect of *EPI*-W and *EPI*-S treatment on iNOS expression in macrophages was confirmed.

As shown in Fig. 2c-d, it was confirmed that the expression of iNOS was increased in the LPS-treated group. Compared to the control group, the expression of iNOS significantly increased in the *EPI*-W- and *EPI*-S-treated groups (100 μg/mL, $P < 0.05$). In addition, it was confirmed that the level of iNOS protein expression was significantly higher in the *EPI*-S-treated group than in the *EPI*-W-treated group ($P < 0.05$). This confirms the above result stating that *EPI*-S treatment produced higher NO than *EPI*-W treatment (Fig. 2b).

Several studies on functional substances with immune-enhancing activity have confirmed the effects of such molecules on the production of NO as well as the expression of iNOS, which are enzymes synthesizing them (Eom et al., 2021; Kim et al., 2022a).

3.3.2. EPIs increased the production of pro-inflammatory cytokines (IL-1b & TNF-a)

It is well-known that TNF-α and IL-1β are key mediators of the inflammatory response. TNF-α is produced by activated macrophages and is important for various physiological responses, such as participating in the defense against bacteria and viruses, promoting apoptosis of tumor cells and repair of injured tissues, and inducing the expression of other inflammatory and immune-modulating mediators (Jiang et al., 2021b). IL-1β belongs to the IL-1 subfamily of cytokines and is an essential cytokine against pathogens, responding to various infections, injuries, and immunological challenges (Lopez-Castejon & Brough, 2011). Due to the functional properties of these cytokines, their effect on cytokine expression is often measured to determine the immune-modulating activity of the bioactive compound (Jiang et al., 2021; Kim et al., 2022a; Lee et al., 2022).

As shown in Fig. 3, EPIs showed a positive effect on the production of the two pro-inflammatory cytokines, and *EPI*-W and *EPI*-S treatment significantly increased the production of TNF-α at all tested concentrations ($P < 0.05$, Fig. 3a). *EPI*-W and *EPI*-S treatment at 100 μg/mL increased the TNF-α production to 15.6 ± 2.7 and 17.1 ± 2.5 ng/mL, respectively. In addition, it was also confirmed that treatment with *EPI*-W and *EPI*-S at 10 μg/mL showed significantly higher TNF-α production compared to the control group ($P < 0.05$). However, it was confirmed that there was no significant difference between the *EPI*-W and the *EPI*-S treatment ($P > 0.05$). Regarding IL-1β (Fig. 3b), a significant increase was confirmed after treatment with 100 μg/mL of *EPI*-W and *EPI*-S ($P <$

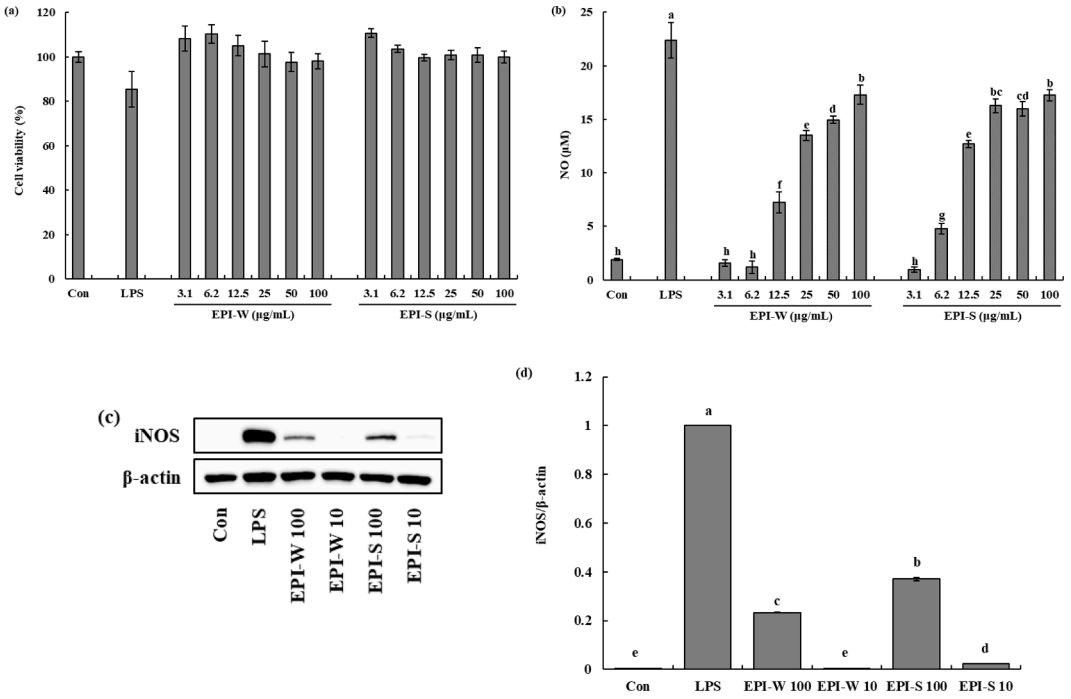


Fig. 2. Effect of EPIs on the cell viability (a), production of NO (b), and expression of iNOS protein (c, d). Values are expressed as the mean ± standard deviation. Different letters (a–h) among samples indicate significant differences by one-way ANOVA followed by Duncan's multiple range test ($P < 0.05$). Con, medium-only treated group; LPS, lipopolysaccharide 1 μg/mL; *EPI*-W 100, *EPI*-W 100 μg/mL; *EPI*-W 10, *EPI*-W 10 μg/mL; *EPI*-S 100, *EPI*-S 100 μg/mL; *EPI*-S 10, *EPI*-S 10 μg/mL.

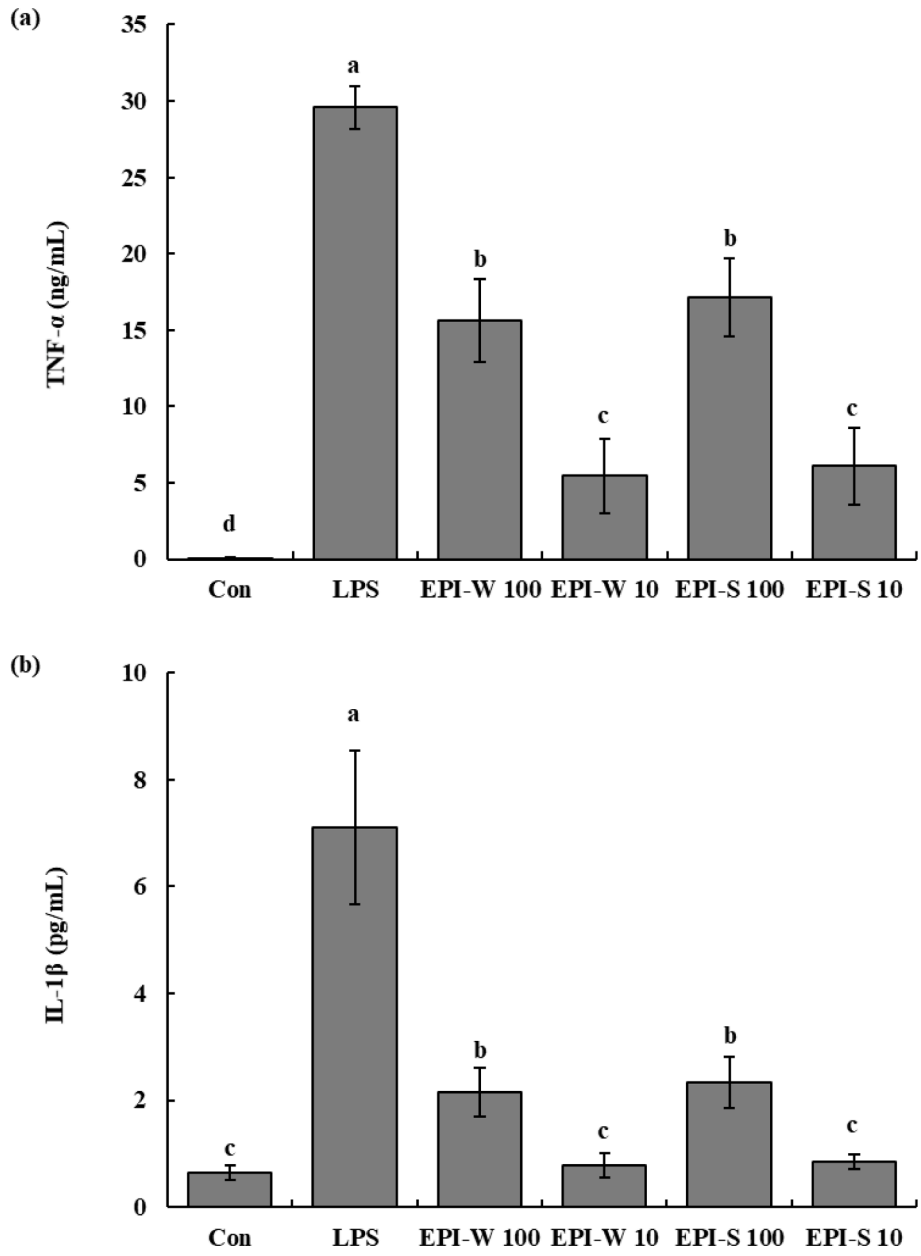


Fig. 3. Effect of EPIs on the expression of pro-inflammatory cytokines TNF-α (a) and IL-1β (b). Values are expressed as the mean ± standard deviation. Different letters (a–d) among samples indicate significant differences by one-way ANOVA followed by Duncan's multiple range test ($P < 0.05$). Con, medium-only treated group; LPS, lipopolysaccharide 1 μg/mL; *EPI*-W 100, *EPI*-W 100 μg/mL; *EPI*-W 10, *EPI*-W 10 μg/mL; *EPI*-S 100, *EPI*-S 100 μg/mL; *EPI*-S 10, *EPI*-S 10 μg/mL.

0.05); meanwhile, it was confirmed that, at a low concentration of 10 μg/mL, there was no effect on the production of IL-1β ($P > 0.05$).

The results showed that both EPIs exert a significant enhancing effect on the production of TNF-α and IL-1β ($P < 0.05$). These findings suggested that the activation of RAW 264.7 macrophages is induced by EPIs treatment, which in turn promotes the pro-inflammatory cytokines production. This finding was also observed in phosvitin from egg yolk protein (Lee et al., 2017), polysaccharide from sea cucumber (Jiang et al., 2021b), water extract from *Hydrangea* leaves (East Asian traditional medicine) (Geum et al., 2020), and ethanol extract from red algae (Wang, Hou, Chiu, & Chen, 2013). These studies reported that immune-enhancing materials effectively increased the production of pro-inflammatory cytokines in macrophages.

3.3.3. EPIs activated the MAPK and NF-κB signaling pathways in RAW 264.7 cells

MAPK and NF-κB signaling pathways are well-known as intracellular signaling pathways, being important for the activation of macrophages (Xu et al., 2022). Macrophages are activated through the MAPK and NF-κB pathways; consequently, the production of inflammatory mediators (NO, TNF-α, and IL-1β) is promoted (Xie et al., 2019). Therefore, to confirm whether the promotion of NO and pro-inflammatory cytokines production was achieved through the two pathways after *EPI* treatment, western blot analysis was performed in this study.

First, it was confirmed that phosphorylation of all three MAPK pathways (including p38, ERK, and JNK pathways) was induced when RAW 264.7 macrophages were treated with *EPI*-W and *EPI*-S (Fig. 4a, 4c–4e). The results demonstrated that the phosphorylation levels of p38, ERK, and JNK were increased in a dose-dependent manner when EPIs were applied ($P < 0.05$). In addition, the p38 and ERK phosphorylation

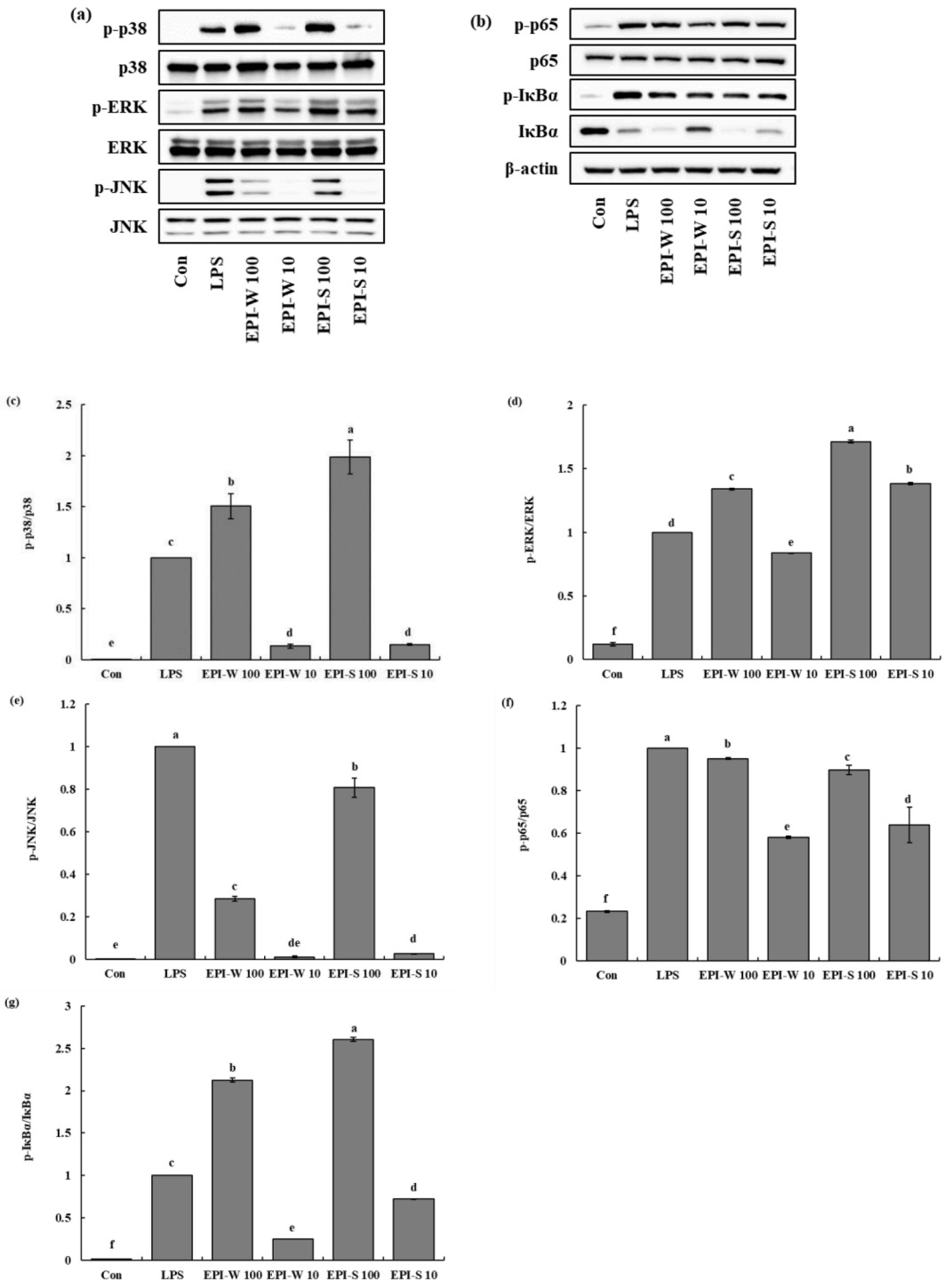


Fig. 4. Effect of EPIs on the activation of MAPK (a, c-e) and NF-κB (b, f, g) signaling pathways in RAW 264.7 macrophages. Values are expressed as the mean ± standard deviation. Different letters (a-f) among samples indicate significant differences by one-way ANOVA followed by Duncan's multiple range test ($P < 0.05$). Con, medium-only treated group; LPS, lipopolysaccharide 1 μg/mL; *EPI*-W 100, *EPI*-W 100 μg/mL; *EPI*-W 10, *EPI*-W 10 μg/mL; *EPI*-S 100, *EPI*-S 100 μg/mL; *EPI*-S 10, *EPI*-S 10 μg/mL.

levels were significantly higher in the 100 μg/mL EPIs treatment than in the LPS treatment group ($P < 0.05$). According to Xu et al. (2022), MAPK pathways regulate the production of NO and cytokines in macrophages by activating the NF-κB signaling pathway. Therefore, to confirm the activation of the NF-κB pathway, the level of p65 protein phosphorylation, a subunit of NF-κB, was determined. As shown in Fig. 4b, EPIs treatment at all tested concentrations induced the phosphorylation of the p65 protein. The results demonstrated that the increase in the phosphorylation level of p65 following EPIs treatment is dose-dependent ($P < 0.05$). This activation of NF-κB is promoted by the phosphorylation and degradation of IκB (Ko, Hyun, Ahn, & Hyun, 2022), a cytoplasmic inhibitor. Thus, the effect of EPIs on IκB phosphorylation was further evaluated and it was revealed that phosphorylation of IκBα occurred in all EPIs concentration treatments.

Our results suggest that EPIs can promote the NO, TNF-α, and IL-1β production in RAW 264.7 macrophages by activating the MAPK and NF-

κB signaling pathways. Similar to our study, egg white protein (Lee, Ahn, & Paik, 2018), exopolysaccharides (Xu et al., 2022), and plant water extract (Ko, Hyun, Ahn, & Hyun, 2022) can also activate macrophages by inducing the phosphorylation of MAPKs. Furthermore, EPIs activate not only the MAPK pathway but also the NF-κB pathway. The degradation of IκBα leads to the dissociation of the IκBα–NF-κB complex, which dissociates NF-κB from the complex; then, NF-κB moves from the cytoplasm to the nucleus, leading to gene transcription and translation (Jiang et al., 2021a). IκBα was phosphorylated by *EPI* treatment; consequently, phosphorylation of p65 was also induced. Consistent with our results, functional substances with immune-enhancing activities can activate macrophages through the NF-κB signaling pathway, thereby promoting the generation of inflammatory mediators (Geum et al., 2020; Xu et al., 2022). In addition, Ge et al. (2023) reported that *Tenebrio molitor* (yellow mealworm) powder can enhance immune function by activating the TLR/NF-κB signaling pathway.

3.3.4. *Blocking assay using specific inhibitors*

To further confirm the mechanism of the immune-enhancing activities of EPIs, a signaling pathway-blocking assay was performed using specific inhibitors. Briefly, RAW 264.7 macrophages were treated with each inhibitor and 100 μg/mL of EPIs for 24 h, followed by an analysis of the NO levels in the supernatant. As shown in Fig. 5, co-treatment with each of the three types of MAPK pathway inhibitors or the NF-κB pathway inhibitor significantly reduced the level of NO induced by *EPI*-W and *EPI*-S treatment ($P < 0.001$). Therefore, blocking the MAPK and NF-κB signaling pathways had a strong negative effect on the NO production in RAW 264.7 macrophages induced by EPIs treatment. These results suggested that EPIs probably induced the activation of RAW 264.7 macrophages through the MAPK and NF-κB signaling pathways.

According to Xie et al. (2019), co-treatment with MAPK and NF-κB pathway inhibitors reduced the activation of RAW 264.7 macrophages induced by polysaccharides from alfalfa and consequently decreased TNF-α production. In addition, phosvitin protein, isolated from egg yolk, activated RAW 264.7 macrophages and increased NO production; however, NO production was decreased when the pathway was blocked using a MAPK, Akt, or NF-κB pathway inhibitor (Lee et al., 2017). Such results are similar to those reported in this work.

4. Conclusion

Edible insect protein isolates (*EPI*-W and *EPI*-S) were obtained from defatted PB larvae. Moreover, the structural and physicochemical properties as well as the immune-enhancing properties of EPIs were investigated. EPIs showed a high content ratio of total essential amino acids, and the secondary structure analysis of EPIs revealed that the β-sheet was the major secondary structure. In addition, the particle size of the dispersion was low in the protein solution state; thus, the solubility of the protein was high. In terms of bio-functional properties, EPIs have been confirmed to exhibit immune-enhancing properties through the activation of macrophages. EPIs promoted the production of NO by increasing the expression of the iNOS protein in RAW 264.7 macrophages. EPIs have also been shown to stimulate the production of the pro-inflammatory cytokines TNF-α and IL-1β. It has been confirmed that the effect on the production of these pro-inflammatory mediators is through the MAPK and NF-κB cell signaling pathways of macrophages. Regarding the immune-enhancing properties, it was confirmed that *EPI*-S showed higher activity than *EPI*-W. Our results suggest that edible insect protein isolated from *P. brevitarsis* larvae can be used as a food source in the future as well as a functional food material that strengthens the immune system of immune-deficient patients and the elderly.

CRediT authorship contribution statement

Jae Hoon Lee: Data curation, Methodology, Visualization, Formal analysis, Validation, Writing – original draft, Writing – review & editing. **Tae-Kyung Kim:** Formal analysis, Visualization. **Yun Jeong Kim:** Methodology, Software. **Min-Cheol Kang:** Visualization, Writing – original draft. **Kyung-Mo Song:** Validation, Writing – original draft, Writing – review & editing. **Bum-Keun Kim:** Conceptualization, Writing – review & editing, Resources, Supervision. **Yun-Sang Choi:** Conceptualization, Validation, Writing – original draft, Writing – review & editing, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

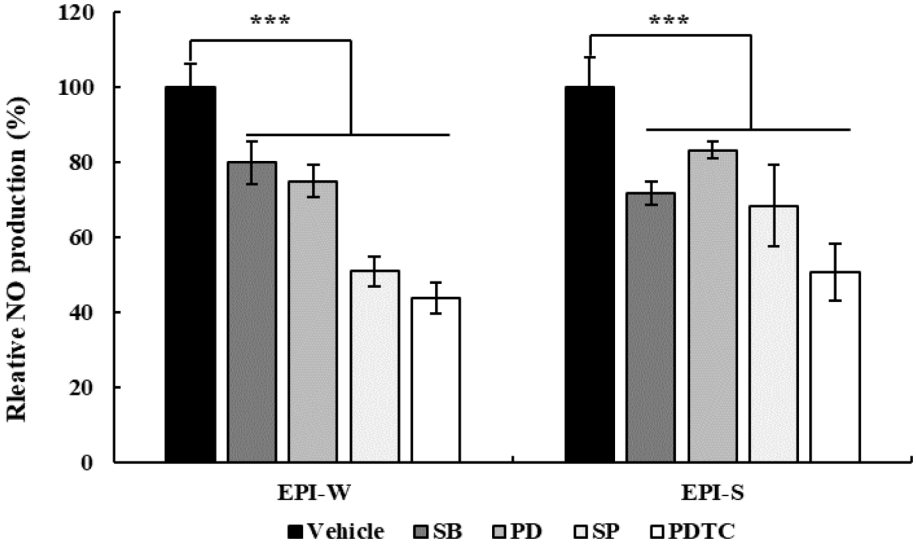


Fig. 5. Effect of MAPK- and NF-κB-specific inhibitors on the NO production induced by EPIs (100 μg/mL) treatment. Values are expressed as the mean ± standard deviation. *** mean statistical difference for $P < 0.001$ (Student's *t*-test) compared with vehicle group. Vehicle, without treatment inhibitor; SB, SB202190 (p38 inhibitor); PD, PD98059 (ERK inhibitor); SP, SP600125 (JNK inhibitor); PDTC, PDTC (NF-κB inhibitor).

Data availability

No data was used for the research described in the article.

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