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Potential anti-inflammatory activity of walnut protein derived peptide leucine-proline-phenylalanine in lipopolysaccharides-irritated RAW264.7 cells

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ABSTRACT

The purpose of this study was to explore the anti-inflammatory mechanism of walnut peptide LPF in the LPS-induced RAW264.7 cells model. The walnut peptide LPF down-regulated the phagocytic ability and the production of nitric oxide (NO) induced by LPS-stimulated RAW264.7. Molecular docking analysis showed that the walnut peptide LPF can bind into the active pocket of inducible nitric oxide synthase (iNOS) with high affinity. Further, the mRNA expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tumour necrosis factor- α (TNF- α) were suppressed by the walnut peptide LPF intervention. Later, we validated the under-expression of iNOS protein by western blot. The decreased mRNA expression of I κ B- α demonstrated the anti-inflammatory effects of the walnut peptide LPF were related to the regulation of the NF- κ B signalling pathway. Our results suggest that the walnut peptide LPF has the potential to be developed as a functional food ingredient against inflammation.

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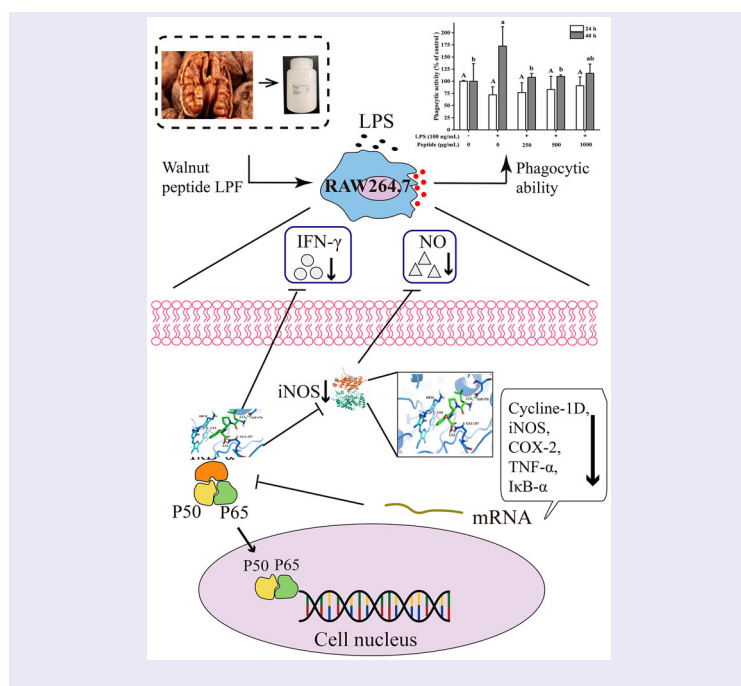
Walnut peptide; anti-inflammation; NF- κ B; iNOS; RAW264.7 cells

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1. Introduction

Inflammation constitutes the intercellular signal cascade. It constitutes the immune defense against harmful microbial invasion and speeds up tissue repair after damage (Leuti et al., 2020). The activation of pattern recognition receptors on immune cells and damage-associated receptor signalling induces massive leukocytes to these aberrant sites. But a prolonged, uncontrolled inflammation state can cause irreversible tissue injuries, chronic inflammatory diseases like rheumatoid diseases, cardiovascular diseases, bowel diseases, and cancer (Alfaddagh et al., 2020). Diet intervention has shown underlying properties to prevent inflammation, the addition of functional food constituents in the diet has been shown improvement in chronic diseases. For example, round scad peptides showed neuroprotective effects on PC₁₂ cells irrigated by glutamate which may be through the interaction with Kelch-like ECH-associated protein-1 (Keap1) (Zhang et al., 2020). The novel anti-inflammatory peptides GPETAFLR from lupine protein hydrolysate conducted a remarkable reduction of TNF, IL-1 β , and CCL2 in THP-1-derived macrophages, meanwhile, it also increased the expression of IL-10, which contributed to the prevention of chronic inflammation. To conclude, the mining and development of novel natural food compounds is a new anti-inflammatory treatment strategy (Businaro et al., 2021; Kim et al., 2021).

Walnut has always occupied an important position in the functional food market due to its high nutritional and functional properties. Multiple functions of walnut such as anticancer, anti-inflammation, antioxidant activity, and several pharmacological activities like brain and cardiovascular protection, diabetes treatment were reported by many kinds of literature (Ni et al., 2021). Walnut protein as one of the main ingredients

in a defatted walnut meal comprised approximately 14.6% of the total mass, is usually used as feed due to inadequate solubility and functional insufficiency (Feng et al., 2019). The by-products with high protein content could be a good source of bioactive peptides. Recently, increasing numbers of bioactive peptides identified from walnut protein have received focus (Lv et al., 2017). Moreover, some researchers reported that walnut peptides could improve oxidative stress, decrease the neuroinflammation caused by excessive pro-inflammatory factors such as neurodegenerative disorder (Daroit & Brandelli, 2021; Wang et al., 2018). Defatted walnut meal hydrolysates protected PC₁₂ cells from H₂O₂-induced oxidative damage and improve memory disturbance in mice (Chen et al., 2015). Walnut peptide EVSGPGLSPN could alleviate the neuroinflammation induced by H₂O₂ via enhancing antioxidant enzyme activity and suppressing the overactivation of NF- κ B and Caspase (Liu et al., 2019).

Mutual crosstalk between peripheral immunity and central neuroinflammation was being found recently. LPS-induced macrophages stimulated the inflammatory response of BV-2 microglia which indicated that peripheral inflammation mediated by macrophages could accelerate the onset of neuroinflammation (Pan et al., 2016). Similarly, inflammatory microglial can recruit peripheral innate immune cells by secreting various chemokines. The complex interactions between these innate immunity cells in the two zones exacerbate the injury of the central nervous system. Therefore, reduction of signalling transmission between the peripheral and central immune systems is an effective strategy for the remission of neurodegenerative diseases. Although the walnut peptide has significant potential in the field of prevention of neuroinflammation, only few reports based on the immunoregulatory effect of walnut peptides on peripheral innate immunity are available. The present study is based on LPF, a low molecular weight peptide, isolated from walnut protein hydrolysates; the low-molecular-weight fraction of walnut protein hydrolysate attenuated the memory impairment through reducing the neuroinflammation and oxidative stress in brain tissue induced by intraperitoneal injection of LPS; after purification and molecular characterization, a total of 18 peptides were identified from the low-molecular-weight fraction of walnut protein hydrolysate, the LPF showed the strong inhibitory effects on proinflammatory mediators by downregulating related enzyme expressions and mRNA expressions in LPS-induced BV-2 microglial cells model (Wang et al., 2020). To further clarify the role of walnut peptide LPF in moderation of peripheral inflammatory innate immune cells, we used the RAW264.7 macrophages as an *in vitro* model to explore the anti-inflammatory properties of LPF. In our research, we investigated the effects of LPF on the phagocytic ability, the change in mRNA relative expression of pro-inflammatory factors such as INOS, COX-2, TNF- α , and I κ B- α , the levels of pro-inflammatory factors (IFN- γ , TNF- α , and IL-6) in LPS-induced RAW264.7 cells, and explored the potential mechanisms of LPF as an inhibitor of NO production.

2. Materials and methods

2.1. Materials and reagents

RAW264.7 mouse macrophage cells were obtained originally from the American type culture collection (ATCC). The peptide was synthesized by Sangon Biotech Co., Ltd.

(Shanghai, China) at >95% purity. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Co. (MD, US). Mouse tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interferon γ (IFN- γ) enzyme-linked immunosorbent assay (ELISA) kits were obtained from ThermoFisher Scientific (Waltham, MA, USA). Lipopolysaccharides (LPS, from *Escherichia coli* O₅₅: B₅) and CCK-8 kit were acquired from Sigma (St. Louis, MO, USA). Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) Apoptosis Detection Kit, Cell Cycle and Apoptosis Analysis Kit were purchased from Yeasen Biotech Co., Ltd. (Shanghai, China). Primary antibodies against iNOS and β -actin were acquired from Abcam (Shanghai, China).

2.2. Cell culture

RAW264.7 cells were cultured in DMEM medium (containing 10% FBS) at 37°C. The cells were cultured in a 5% (v/v) CO₂ incubator and digested with 0.25% (w/v) trypsin before experiment.

2.3. Determination of cell viability, apoptosis and phagocytic ability

RAW264.7 cells were performed as described as reference Yi et al. (2020). The cells were trypsinized and reseeded in a 96-well plate adjusted to a density of 6×10^4 cells/mL. After plating, cells were stimulated by indicated concentration of LPS (100 ng/mL) and peptides (0, 250, 500, 1000 μ g/mL) for 24 h or 48 h culture. PBS was used in the control group. The cell viability assay was performed using a CCK-8 kit (Sigma, USA) following the manufacturer's instructions. The phagocytic ability was detected by the neutral red uptake assay described in Wang et al. (2016). Cell apoptosis was determined using flow cytometry by Cell Cycle and Apoptosis Analysis Kit (Yeasten, China) according to the manufacturer's guidance at 24 h.

2.4. Evaluation of NO

The content of NO was determined as described previously with minor modifications (Payoungkiattikun et al., 2020). Cells were seeded in a 96-well plate (1×10^4 cells/well) and cultured until 80% confluence. 100 ng/mL of LPS with PBS or peptide (250–1000 μ g/mL) co-incubated for 24 h or 48 h at 37 °C. Then, an equal volume of Griess reagent was mixed with medium, and then cultured in submerged static culture for 10 min at room temperature. The outcome measurements were obtained by a microplate reader at 540 nm.

2.5. Molecular docking

The 3D structure of iNOS (PDB ID: 3E6T), downloaded from the Protein Structure Database (Protein Data Bank, PDB) (<https://www1.rcsb.org/>), was pretreated by removing the duplicate chain, water, and ligands, adding polar hydrogen atoms and computing gasteiger charges using AutoDock Tools software (ADT, version 1.5.6) (Morris et al., 2009). The 3D structure of walnut peptide LPF as the ligand was built and optimized

by the GAFF force field using Avogadro (Hanwell et al., 2012). Before the docking analysis, the LPF was performed the same preprocessing including the addition of polar hydrogen atoms and computation of gasteiger charges, and then the roots and the rotatable bonds were automatically defined in AutoDock Tools software. The coordination of co-crystallized inhibitor (ligand: 1A2) was chosen as the centre of grid box (X: 123.56, Y: 114.68, Z: 35.75), the docking search area was set at 20 Å with a grid resolution of 1.00 size. Autodock Vina was used to searching the best binding sites (Trott & Olson, 2009). The best-docked conformation of ligand was chosen and visualized using PYMOL version 1.8.2.2 and Discovery Studio 2016.

2.6. RT-qPCR analysis

RAW264.7 cells were seeded in a 24-well culture plate overnight. LPS (100 ng/mL) was added for an additional 24 h after 2 h of different concentrations of peptides (0, 250, 500, 1000 µg/mL) treated. Total RNA of samples was extracted using the E.Z.N.A.® Total RNA Kit II (Omega Bio-Tek, USA). The reverse transcription of RNA to cDNA was performed using the HiScript® II qRT Super kit (Vazyme Biotech, USA). The SYBR Green I kit (Vazyme Biotech, USA) with the Light Cycler® 480 II system (Roche, USA) was performed to the real-time quantitative PCR analysis. The reaction mixture was prepared for PCR at 95°C for 30 s, then performed to 40 cycles of denaturation at 95°C for 10 s, annealing and extension at 60°C for 30 s. The expression of GAPDH was served as the internal standard for calibration. The qPCR primers are listed in Table 1. $2^{-\Delta\Delta CT}$ method was calculated the relevant changes of mRNA expression.

2.7. Western blot analyses

RAW264.7 cells were cultured as indicated above. The total protein content of the cell lysis buffer was measured by the BCA protein assay kit. Samples were subjected to 15% SDS-PAGE, and then transferred to polyvinylidene fluoride membranes (PVDF) after. PVDF was incubated at 4°C with primary antibody overnight before blockage, and then placed with alkaline phosphatase-conjugated secondary antibodies at room temperature. The relative expression of protein was normalized by β -actin via Image J applicant.

Table 1. The qPCR primers.

Gene	FP	RP	Reference
iNOS	GTGTTCCACCAGGAGATGTTG	CTCCTGCCCACTGAGTCCGTTT	http://www.rsc.org/suppdata/c9/nr/c9nr03060c/c9nr03060c1.pdf
COX-2	TGAGCAACTATTCCAACCAGC	GCACGTAGTCTTCGATCACTATC	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3419084/
TNF- α	GCCACGTCGTAGCAAACCAC	GCAGGGGCTCTTGACGGCAG	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5413058/table/pone.0176823.t003/
IkB α	TGAAGGACGAGGAGTACGAGC	TTCGTGGATGATTGCCAAGTG	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3419084/table/T1/
Cyclin D1	GCCCTCCGTGTCTTACTTCA	CTCTTCGCACTTCTGCTCCT	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4423449/

2.8. Measurement of pro-inflammatory factors

RAW264.7 cells were cultured following the method described by Sui et al. (2020). In short, RAW264.7 cells were plated in a 24-well culture plate and performed 12 h of incubation for adherence. Then, peptides LPF (1000 µg/mL) were added. 2 h after peptides addition, 100 ng/mL LPS was added for 24 h. The pro-inflammatory factors (IFN-γ, TNF-α, IL-6) were measured by ELISA kits.

2.9. Statistical analysis

Statistical analysis was performed with SPSS 19.0. Data are shown as means ± standard deviation. Multiple comparison tests were performed using one-way analysis of variance (ANOVA), significant differences ($p < 0.05$) between groups were evaluated by Student t-test.

3. Results

3.1. Effect of LPF on cell viability, apoptosis and phagocytic ability of macrophages

As shown in Figure 1A, the effects of walnut peptide LPF on the viability of RAW264.7 macrophages induced by LPS were determined based on the CCK-8 method. Cell viability did not significantly affect by LPS at 24 or 48 h, and there were no significant changes in walnut peptides treatment. The apoptotic rates were largely enhanced in LPS-treated RAW264.7 cells, but the walnut peptide LPF had no effects on preventing apoptosis at 24 h (Figure 1B). The results of phagocytic ability were observed in Figure 1C. LPS increased significantly the phagocytic ability of RAW264.7 cells after 48 h compared with control. For the treatment groups, 250 and 500 µg/mL of peptides inhibited the enhanced phagocytic activities by nearly 37.38% and 36.19%, respectively. Additionally, we determined the gene expression of Cyclin D1 to observe the potential influences of walnut peptide LPF on the cell cycle. As evident from Figure 1D, the 500 and 1000 µg/mL of walnut peptide LPF significantly suppressed the increased expression of Cyclin D1 induced by LPS, the rates of inhibition were 29.87% and 37.89%, respectively.

3.2. Effects of walnut peptide LPF on the production of NO

The influences of LPS and walnut peptide LPF on the content of NO were showed in Figure 2, the level of NO in the LPS treated group was upregulated nearly 2-fold at 24 h, but no significant changes after walnut peptide LPF treatment was observed. At 48 h, the LPS treated group still exhibited a higher expression level than the PBS group ($p < 0.001$), while walnut peptide LPF treated groups all showed significant down-regulation of NO (24.94%, 23.4%, and 20.73%, respectively). To investigate the mechanism of walnut peptides affecting the production of NO, we further performed the molecular docking analysis and determined the mRNA levels of iNOS in LPS-induced RAW264.7 cells.

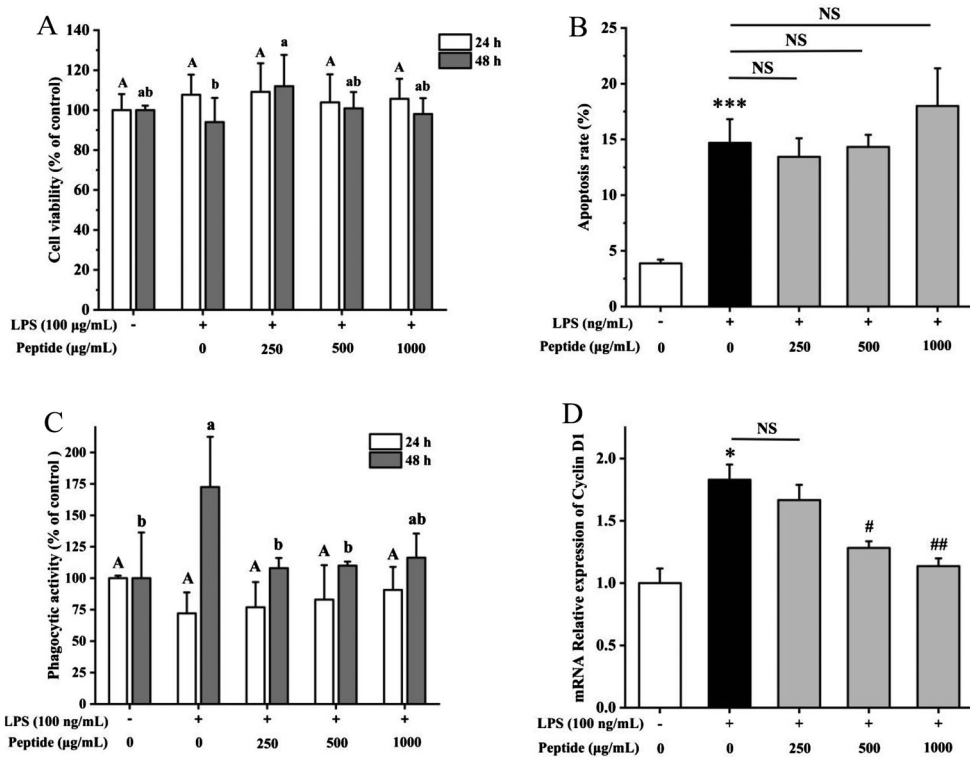


Figure 1. Effects on cell viability (A), apoptosis (B), phagocytic ability (C) and mRNA expression of Cyclin D1 (D) in RAW264.7 cells. Results of cell viability and phagocytic ability were expressed as a percentage of the respective control. The results were expressed as the mean $\pm$ SD. * indicated $p < 0.05$; *** indicated $p < 0.001$ compared with the control group; # indicated $p < 0.05$, ## indicated $p < 0.01$; NS indicated no significant changes compared with the LPS group. Uppercase letters indicated differences for 24 h; lowercase letters denoted differences for 48 h ($p < 0.05$).

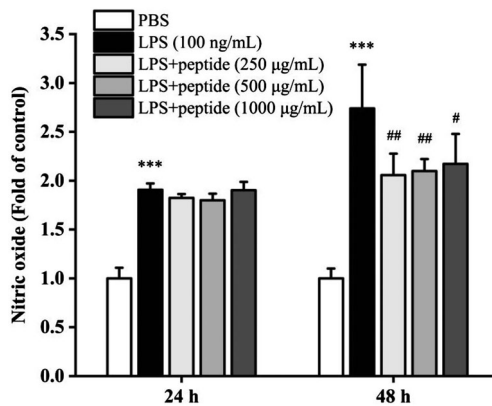


Figure 2. NO production in LPS-induced RAW264.7 cells at 24 and 48 h of treatment with walnut peptides. The results were expressed as the mean $\pm$ SD. *** indicated $p < 0.001$ compared with control group; # indicated $p < 0.05$; ## indicated $p < 0.01$ compared with LPS group.

3.3. Molecular docking analysis

Based on the inhibitory ability of NO, we speculated walnut peptide LPF might be a potential iNOS inhibitor. The interactions between walnut peptide LPF and the iNOS were shown in Figure 3. The main interactions included van der Waals, conventional hydrogen bond, and pi-alkyl. Figure 3B showed that two hydrogen bond interactions between the key active sites of iNOS including Leu and Asp-376, Phe, and Glu-257; at the same time, Phe also formed a pi-pi stacked bond to the cofactor HEM, which plays a pivotal role in the electron transport chain. Other bonds such as pi-alkyl and alkyl were shown in Figure 3C, these simulation results firmed our speculation that the walnut peptide LPF would be a strong inhibitor of NO.

3.4. Effects of walnut peptide LPF on expression changes of pro-inflammatory and NF- κ B signalling factors in LPS-induced RAW264.7 cells

We assayed the expression of pro-inflammatory factors associated with LPS-stimulation. As shown in Figure 4A and 4C, the mRNA and protein expression of iNOS in the LPS

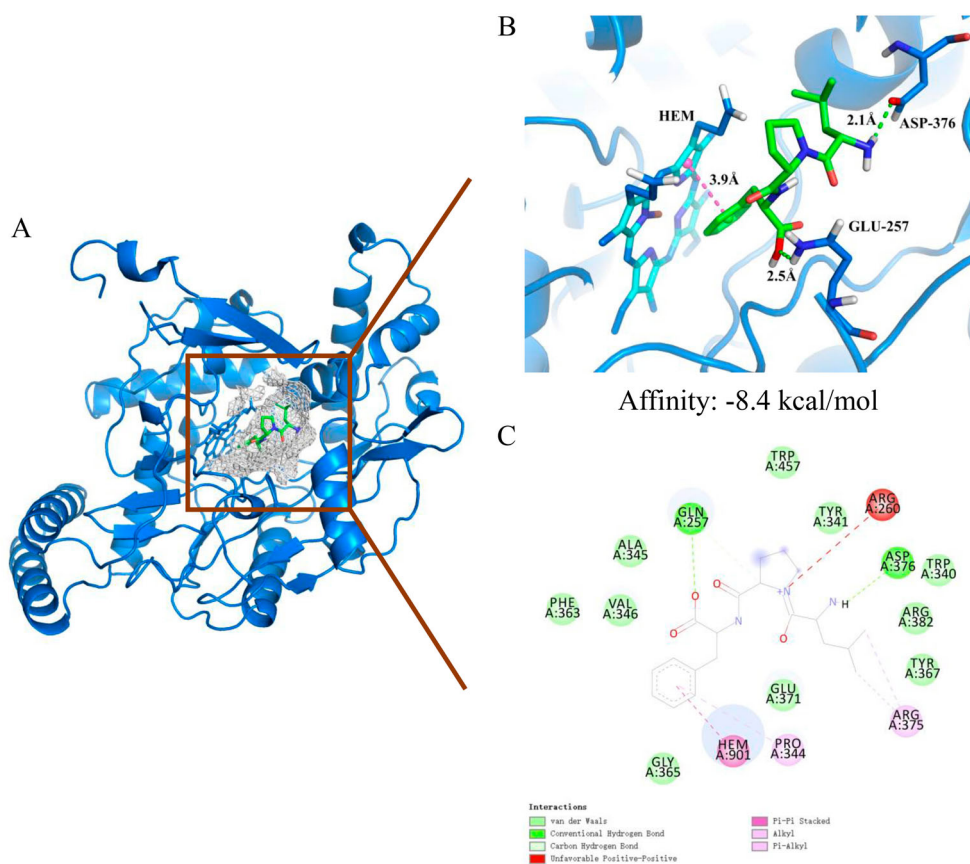


Figure 3. The 3D molecular interactions of the walnut peptide LPF into the active sites of iNOS (A). 3D models of the interactions generated by PYMOL in iNOS active sites (B), only hydrogen-bonding and pi-pi stacked were labelled by green and pink dashes. The 2D molecular interactions of the walnut peptide LPF with iNOS generated by Discovery Studio Client 2016 (C).

treated group was upregulated significantly compared with the control, and the 1000 $\mu\text{g}/\text{mL}$ of walnut peptide LPF showed the highest inhibitory rate in mRNA expression of iNOS ($p < 0.01$). Likewise, the 1000 $\mu\text{g}/\text{mL}$ of walnut peptides LPF significantly suppressed the increment of COX-2 (Figure 4B), with the inhibitory rate of 30.68%. The mRNA expression of TNF- α and I κ B- α were measured by RT-qPCR, as indicated in

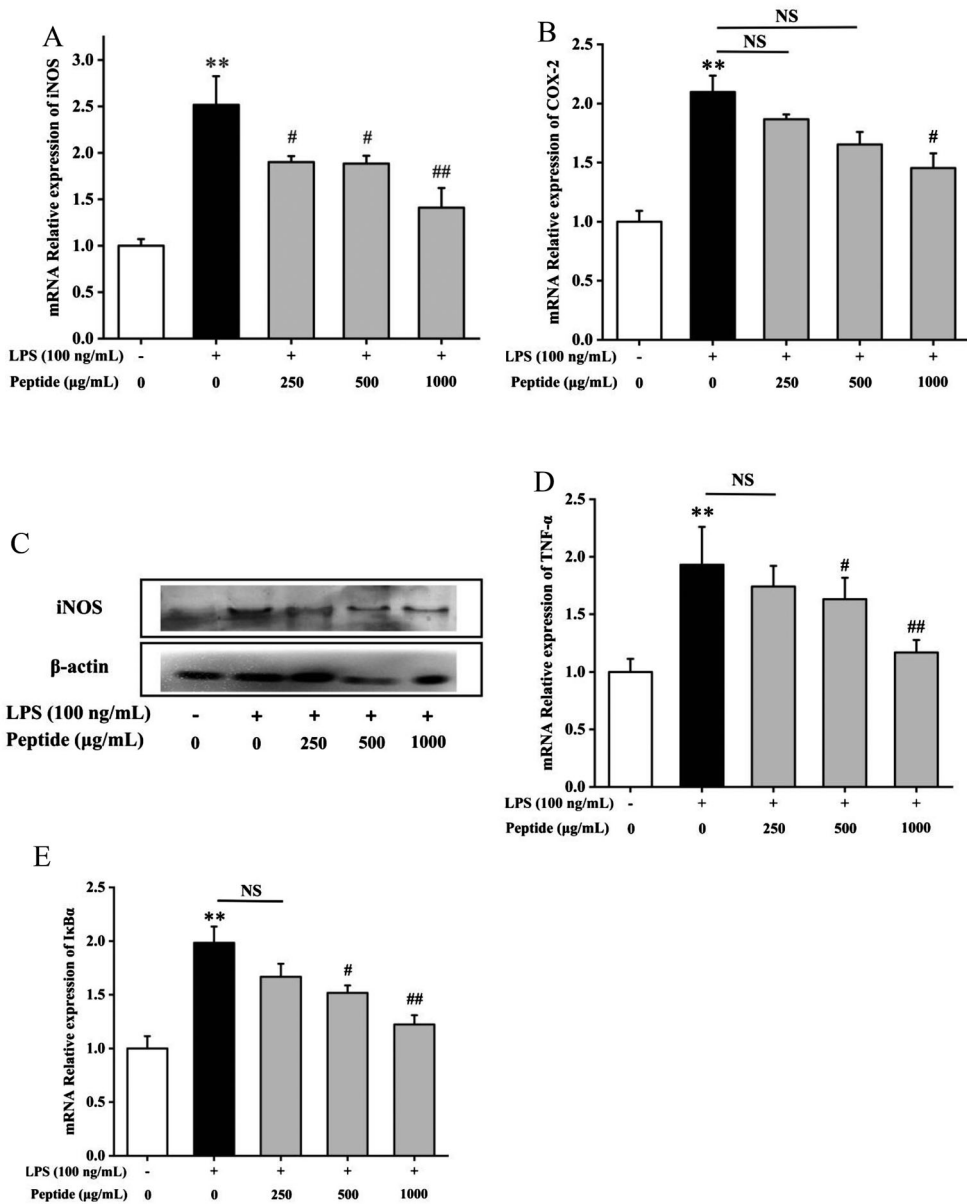


Figure 4. Effects of walnut peptide LPF on iNOS (A), COX-2 (B) encoding gene mRNA expression, protein expression of iNOS (C), mRNA relative expression of TNF- α (D) and I κ B- α (E) in LPS-induced RAW264.7 cells. The results were expressed as the mean $\pm$ SD. ** indicated $p < 0.01$ compared with control group; # $p < 0.05$, ## $p < 0.01$; N.S., no significance compared with LPS group.

Figure 4D and 4E, the 1000 $\mu\text{g/mL}$ of peptide LPF demonstrated the highest inhibitory activity, the inhibitory percents were 39.38% and 38.32%, respectively. $\text{I}\kappa\text{B-}\alpha$ is a direct indicator of NF- κB signalling activation, it can be inferred that walnut peptide LPF downregulated the mRNA expression of pro-inflammatory factors by inhibiting upstream NF- κB signalling pathways.

3.5. Influences of the production of IFN- γ , TNF- α and IL-6 in LPS-induced RAW264.7 cells

According to the results of expression of pro-inflammatory genes, the 1000 $\mu\text{g/mL}$ treatment group showed the best anti-inflammatory activity. Therefore, the concentration was chosen to perform the ELISA experiments. Figure 5A showed that the walnut peptide LPF significantly reduced the level of IFN- γ compared with the LPS treatment group, the inhibitory rate was 12.73%. while the levels of TNF- α and IL-6 were remarkably elevated compared with the LPS group (Figure 5B and 5C), which displayed pro-inflammatory phenotypes. The level change of TNF- α was against gene expression, it might indicate the presence of post-transcriptional regulation of it.

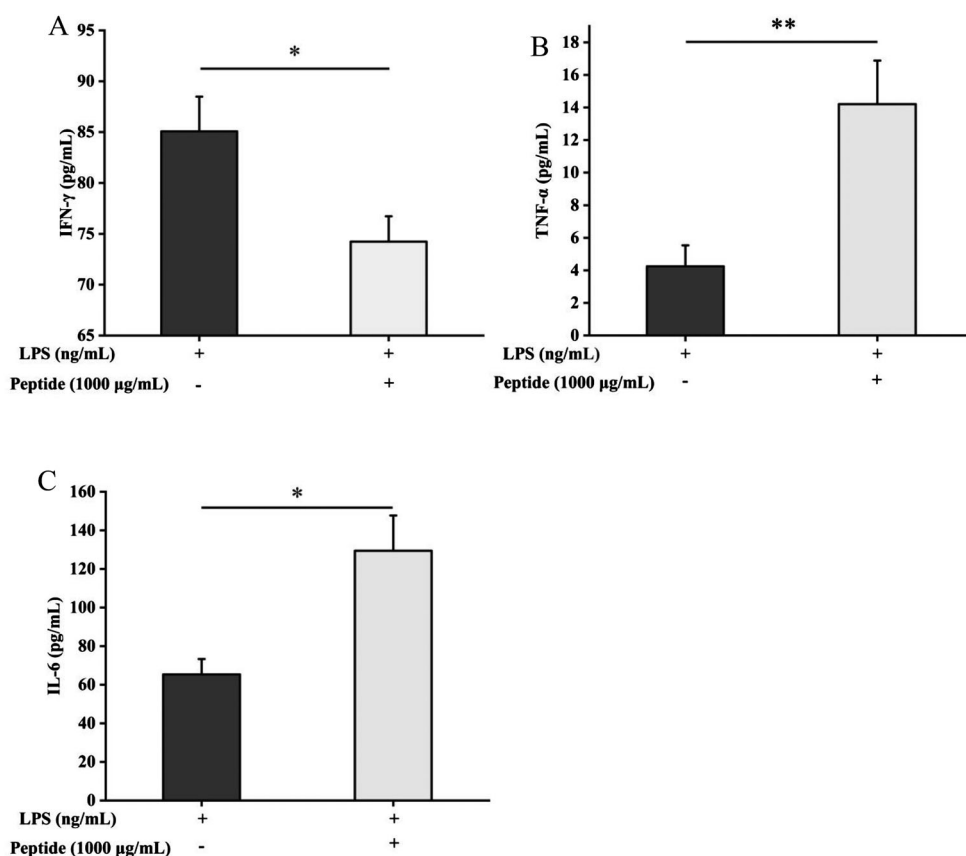


Figure 5. The effects of walnut peptide LPF on IFN- γ (A), TNF- α (B), IL-6 (C) level in LPS-induced RAW264.7. The results were expressed as the mean $\pm$ SD. * indicated $p < 0.05$; ** indicated $p < 0.01$ compared with LPS group.

4. Discussion

Walnuts as a functional food have attracted much attention in health-promotion, walnut-enriched diet can reduce the level of inflammatory factors *in vivo* (Atanasov et al., 2018). Bioactive peptides from walnut protein have become a focus of functional gradient research. Bioactive peptides are usually consisting of 2–20 amino acids, existing in protein, with low cost, high efficiency, absorbability, and nontoxicity (Ulug et al., 2021). Short peptides derived from walnut showed various bioactivity, especially the protection of neuroinflammation along with oxidative stress. The tripeptide LPF displayed strong anti-neuroinflammatory effects (Wang et al., 2020). The tripeptide was composed of three hydrophobic amino acids, high hydrophobicity benefited for the peptide to cross the cellular membrane (Vo et al., 2013). As reported, Leucine can attenuate the LPS-induced inflammatory responses *in vitro* and *in vivo* through inhibited TLR₄-MyD88 signalling and upregulated the expression of IL-10 (Giri et al., 2016). Additionally, the proline-rich polypeptide manifests anti-inflammatory activity through the inhibition of NF- κ B activation (Zabłocka et al., 2010). Thus, the amino acid composition of peptides might be critical for the anti-inflammatory property. Macrophages could be activated by LPS to increase phagocytic potential (Markov et al., 2020). As shown in Figure 1C, LPF suppressed the increment of phagocytic ability caused by LPS. Cyclins are the key proteins involved in cell cycle progression, Cyclin D1 is responsible for G1/S phase transition (Pawlonka et al., 2021). Our results showed that the 500 and 1000 μ g/mL of peptide LPF down-regulated the Cyclin D1 mRNA expression (Figure 1D). Therefore, the LPF might inhibit the excessive cell proliferation stimulated by LPS, thus influence the proliferation and differentiation of macrophages.

NO is an important inflammation mediator produced by inducible nitric oxide synthase (iNOS). iNOS, mainly mediated by the NF- κ B signalling pathway, is usually inactive at a steady-state and activated to synthesize NO when macrophages are stimulated by TNF- α , LPS, and IL-1 β (Wen et al., 2016). A long-term high level of NO has been confirmed to cause endotoxemia and severe inflammation (Moon et al., 2019). To inhibit LPS-induced NO generation is one of the main strategies to attenuate the inflammation. Computer-aided molecular docking has been widely used to study the interactions between natural production and inflammatory mediator. The walnut peptide LPF resulted in a significant decrease in the level of NO at 48 h (Figure 2). The docking results showed the LPF formed strong interactions with INOS protein (Figure 3). The residue Arg-375 is a key amino acid to regulate H₄B which contributes to electron transfer and stability of dimer (Cinelli et al., 2020). Cofactor heme (HEM 901) is the catalytic centre that oxidizes L-arginine to L-citrulline, it also serves as an anchor and plays a key role in the electron transfer chain (Bignon et al., 2019). Additionally, it has been reported that Arg-260 and Gln-257 were the targeted residues of novel NO inhibitors (Wang et al., 2017). In our results, the LPF could fit in the active pocket, and form stable molecular bonds with these critical residues and heme, thus it has the potential to be a NO inhibitor.

The level of pro-inflammatory factors was regulated by multiple signalling pathways. *Rodgersia podophylla* leaves extracts decreased the pro-inflammatory mediators NO and iNOS via degradation of I κ B- α and nuclear accumulation of p65 (Kim et al., 2020). NF- κ B is a key signalling pathway to respond the external stimulation, it is involved in cell proliferation, apoptosis, inflammation, and diverse cellular activities. NF- κ B is activated

when LPS specially binds to TLR₄ (Q. Zhang et al., 2017). IκB-α is one of the inhibitors of κB assembled from three members, IκB kinase β (IKKβ) is phosphorylated and activated in classical pathway, subsequently phosphorylates IκB-α and contributes to IκB-α-p50-p65 polymer breakage and p50/p65 subunits nuclear translocation (Zinatizadeh et al., 2021). After that, IκB-α was quickly expressed to form a negative feedback loop, making it to be an ideal indicator for NF-κB signalling activation. In the present study, the 500 and 1000 µg/mL of LPF treatment groups significantly reduced the mRNA expressions of IκB-α in LPS-induced RAW264.7 cells (Figure 4E). Similar results were reported in other food-derived peptides (Ren et al., 2018; Yi et al., 2020). Cyclooxygenase enzymes-2 (COX-2) is an essential rate-limiting enzyme involved in catalyzing the biosynthesis of pro-inflammatory factor prostaglandin E2 (PGE2) from arachidonic acid (Xue et al., 2020). In this study, the mRNA expressions of INOS, COX-2, TNF-α, and the protein content of INOS were down-regulated in LPF treatment groups (Figure 4A-4D). These changes in pro-inflammatory factors might be linked to the decrease in mRNA expressions of IκB-α.

IFN-γ is also regulated by the NF-κB signalling pathway in macrophages and is important in macrophage activation, pro-inflammatory, the resistance of pathogens, promoting T cells differentiate to Th1 cells, and antitumour (Guo et al., 2021). It can differentiate unactivated macrophages into classically activated pro-inflammatory macrophages (Type M1). In the present research, the content of IFN-γ induced by LPS was significantly decreased by treating with the 1000 µg/mL of walnut peptide LPF (Figure 5A). It demonstrated that the walnut peptide LPF probably played protective effects against inflammation via preventing polarization of M1 type macrophages, subsequently, inhibited excessive Th1-type immune response. Based on the results, further exploration was needed to explain the phenomenon. TNF-α is the acute inflammatory factor causing systematic inflammatory. The present results demonstrated that the mRNA level of TNF-α was reduced in the 1000 µg/mL of walnut peptide LPF treatment group (Figure 4D), we speculated that the peptide LPF exerted the anti-inflammatory effects by inhibiting activation of NF-κB signalling pathway. However, the opposite results were noted for the cytokine production, the level of TNF-α and IL-6 was significantly higher in the treatment group than in the LPS group (Figure 5B, C). The changes in protein content are determined by mRNA concentration, translation efficiency, and degradation of the existing protein, there is not a linear relationship between the mRNA content and protein (de Sousa Abreu et al., 2009). Lahtvee et al. (2017) quantified protein and mRNA abundances to demonstrate the discrepancy of translation efficiency is exist in eukaryotic cell *Saccharomyces cerevisiae*. The mature progress of proteins also needs complicated post-translational modification, it can affect the consistency between the tendency of mRNA and protein. Besides, the changes of mRNA and protein may have different time windows. For the assays of iNOS and NO, there was no obvious effect on the content of NO at 24 h, even though the mRNA expression of iNOS was suppressed (Figure 2). Moreover, TNF-α and IL-6 were regulated by NF-κB, nuclear factor of T cell (NEAT), activation protein-1 (AP-1) signalling pathways, the complex signal transduction and different delivery velocity of pathways also lead to the results (Kaur et al., 2020; Mahdavi Sharif et al., 2020).

The above results demonstrated that the walnut peptide LPF exerted the anti-inflammatory effects through regulation of the inflammatory signalling pathway and inhibition

of cytokines level in LPS-induced RAW264.7 cells. Whereas, the *in vitro* model cannot mock the complex disease state due to the lack of signalling substances present in inflamed tissue such as protein, lipid, and lipoproteins. Due to this, further animal experiments were required to validate and investigate the detailed anti-inflammatory mechanism of walnut peptide LPF.

5. Conclusions

The research investigated the anti-inflammatory effects and mechanisms of walnut peptide LPF in the LPS-irritated RAW264.7 cells. The walnut peptide LPF regulated the phagocytic ability and the mRNA expression of Cyclin D1 in LPS-induced RAW264.7 cells. Molecular docking results showed that the LPF can effectively bind to active sites of INOS. Moreover, the walnut peptide LPF down-regulated the mRNA expression of inflammatory mediators (such as INOS, COX-2, and TNF- α) and the level of IFN- γ via inhibition of the NF- κ B signalling pathway. Of all treatment groups, the 1000 μ g/mL of walnut peptide LPF showed the best inhibitory activity. In conclusion, our finding indicated that the walnut peptide LPF could be applied in functional food ingredients in health promotion to regulate inflammation-associated diseases.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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References

- Alfaddagh, A., Martin, S. S., Leucker, T. M., Michos, E. D., Blaha, M. J., Lowenstein, C. J., Jones, S. R., & Toth, P. P. (2020). Inflammation and cardiovascular disease: From mechanisms to therapeutics. *American Journal of Preventive Cardiology*, 4, 100130. <https://doi.org/10.1016/j.ajpc.2020.100130>
- Atanasov, A. G., Sabharanjak, S. M., Zengin, G., Mollica, A., Szostak, A., Simirgiotis, M., Huminiecki, Ł., Horbanczuk, O. K., Nabavi, S. M., & Mocan, A. (2018). Pecan nuts: A review of reported bioactivities and health effects. *Trends in Food Science & Technology*, 71, 246–257. <https://doi.org/10.1016/j.tifs.2017.10.019>
- Bignon, E., Rizza, S., Filomeni, G., & Papaleo, E. (2019). Use of computational biochemistry for elucidating molecular mechanisms of nitric oxide synthase. *Computational and Structural Biotechnology Journal*, 17, 415–429. <https://doi.org/10.1016/j.csbj.2019.03.011>
- Businaro, R., Vauzour, D., Sarris, J., Munch, G., Gyengesi, E., Brogelli, L., & Zuzarte, P. (2021). Therapeutic opportunities for food supplements in neurodegenerative disease and depression. *Frontiers in Nutrition*, 8, 669846. <https://doi.org/10.3389/fnut.2021.669846>
- Chen, H., Zhao, M., Lin, L., Wang, J., Sun-Waterhouse, D., Dong, Y., Zhuang, M., & Su, G. (2015). Identification of antioxidative peptides from defatted walnut meal hydrolysate with potential for improving learning and memory. *Food Research International*, 78, 216–223. <https://doi.org/10.1016/j.foodres.2015.10.008>

- Cinelli, M. A., Do, H. T., Miley, G. P., & Silverman, R. B. (2020). Inducible nitric oxide synthase: Regulation, structure, and inhibition. *Medicinal Research Reviews*, 40(1), 158–189. <https://doi.org/10.1002/med.21599>
- Daroit, D. J., & Brandelli, A. (2021). *In vivo* bioactivities of food protein-derived peptides – a current review. *Current Opinion in Food Science*, 39, 120–129. <https://doi.org/10.1016/j.cofs.2021.01.002>
- de Sousa Abreu, R., Penalva, L. O., Marcotte, E. M., & Vogel, C. (2009). Global signatures of protein and mRNA expression levels. *Molecular BioSystems*, 5(12), 1512–1526. <https://doi.org/10.1039/b908315d>
- Feng, L., Peng, F., Wang, X., Li, M., Lei, H., & Xu, H. (2019). Identification and characterization of antioxidative peptides derived from simulated *in vitro* gastrointestinal digestion of walnut meal proteins. *Food Research International*, 116, 518–526. <https://doi.org/10.1016/j.foodres.2018.08.068>
- Giri, S. S., Sen, S. S., Jun, J. W., Sukumaran, V., & Park, S. C. (2016). Protective effects of leucine against lipopolysaccharide-induced inflammatory response in *Labeo rohita* fingerlings. *Fish & Shellfish Immunology*, 52, 239–247. <https://doi.org/10.1016/j.fsi.2016.03.148>
- Guo, C., Bi, J., Li, X., Lyu, J., Liu, X., Wu, X., & Liu, J. (2021). Immunomodulation effects of polyphenols from thinned peach treated by different drying methods on RAW264.7 cells through the NF- κ B and Nrf2 pathways. *Food Chemistry*, 340, 127931. <https://doi.org/10.1016/j.foodchem.2020.127931>
- Hanwell, M., Curtis, D. E., Lonie, D. C., Vandermeersch, T., Zurek, E., & Hutchison, G. R. (2012). Avogadro: An advanced semantic chemical editor, visualization, and analysis platform. *Journal of Cheminformatics*, 4(1), 17. <https://doi.org/10.1186/1758-2946-4-17>
- Kaur, S., Bansal, Y., Kumar, R., & Bansal, G. (2020). A panoramic review of IL-6: Structure, pathophysiological roles and inhibitors. *Bioorganic & Medicinal Chemistry*, 28(5), 115327. <https://doi.org/10.1016/j.bmc.2020.115327>
- Kim, H. N., Kim, J. D., Park, S. B., Son, H. J., Park, G. H., Eo, H. J., Kim, H. S., & Jeong, J. B. (2020). Anti-inflammatory activity of the extracts from *Rodgersia podophylla* leaves through activation of Nrf2/HO-1 pathway, and inhibition of NF- κ B and MAPKs pathway in mouse macrophage cells. *Inflammation Research*, 69(2), 233–244. <https://doi.org/10.1007/s00011-019-01311-2>
- Kim, J. G., Dong, X., Park, S. H., Bayazid, A. B., Jeoung, S. A., & Lim, B. O. (2021). Bioconversion of black rice and blueberry regulate immunity system through regulation of MAPKs, NF- κ B in RAW264.7 macrophage cells. *Food and Agricultural Immunology*, 32(1), 471–481. <https://doi.org/10.1080/09540105.2021.1956434>
- Lahtvee, P. J., Sánchez, B. J., Smialowska, A., Kasvandik, S., Elseman, I. E., Gatto, F., & Nielsen, J. (2017). Absolute quantification of protein and mRNA abundances demonstrate variability in gene-specific translation efficiency in yeast. *Cell Systems*, 4(5), 495–504.e5. <https://doi.org/10.1016/j.cels.2017.03.003>
- Leuti, A., Fazio, D., Fava, M., Piccoli, A., Oddi, S., & Maccarrone, M. (2020). Bioactive lipids, inflammation and chronic diseases. *Advanced Drug Delivery Reviews*, 159, 133–169. <https://doi.org/10.1016/j.addr.2020.06.028>
- Liu, C., Guo, Y., Zhao, F., Qin, H., Lu, H., Fang, L., Wang, J., & Min, W. (2019). Potential mechanisms mediating the protective effects of a peptide from walnut (*Juglans mandshurica* Maxim.) against hydrogen peroxide induced neurotoxicity in PC12 cells. *Food & Function*, 10(6), 3491–3501. <https://doi.org/10.1039/C8FO02557F>
- Lv, Y., Wei, K., Meng, X., Huang, Y., Zhang, T., & Li, Z. (2017). Separation and identification of iron-chelating peptides from defatted walnut flake by nanoLC-ESI-MS/MS and de novo sequencing. *Process Biochemistry*, 59, 223–228. <https://doi.org/10.1016/j.procbio.2017.05.010>
- Mahdavi Sharif, P., Jabbari, P., Razi, S., Keshavarz-Fathi, M., & Rezaei, N. (2020). Importance of TNF-alpha and its alterations in the development of cancers. *Cytokine*, 130, 155066. <https://doi.org/10.1016/j.cyto.2020.155066>
- Markov, A. V., Sen'kova, A. V., Babich, V. O., Odarenko, K. V., Talyshchev, V. A., Salomatina, O. V., Salakhutdinov, N. F., Zenkova, M. A., & Logashenko, E. B. (2020). Dual effect of soloxolone

- methyl on LPS-induced inflammation *in vitro* and *in vivo*. *International Journal of Molecular Sciences*, 21(21), 7876. <https://doi.org/10.3390/ijms21217876>.
- Moon, S. W., Ahn, C. B., Oh, Y., & Je, J. Y. (2019). Lotus (*Nelumbo nucifera*) seed protein isolate exerts anti-inflammatory and antioxidant effects in LPS-stimulated RAW264.7 macrophages via inhibiting NF- κ B and MAPK pathways, and upregulating catalase activity. *International Journal of Biological Macromolecules*, 134, 791–797. <https://doi.org/10.1016/j.ijbiomac.2019.05.094>
- Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., & Olson, A. J. (2009). Autodock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry*, 30(16), 2785–2791. <https://doi.org/10.1002/jcc.21256>
- Ni, Z.-J., Zhang, Y.-G., Chen, S.-X., Thakur, K., Wang, S., Zhang, J.-G., Shang, Y.-F., & Wei, Z.-J. (2021). Exploration of walnut components and their association with health effects. *Critical Reviews in Food Science and Nutrition*. <https://doi.org/10.1080/10408398.2021.1881439>.
- Pan, Y., Shen, B., Gao, Q., Zhu, J., Dong, J., Zhang, L., & Zhang, Y. (2016). Caspase-1 inhibition attenuates activation of BV2 microglia induced by LPS-treated RAW264.7 macrophages. *Journal of Biomedical Research*, 30(3), 225–233. <https://doi.org/10.7555/JBR.30.20150141>
- Pawlonka, J., Rak, B., & Ambroziak, U. (2021). The regulation of cyclin D promoters – review. *Cancer Treatment and Research Communications*, 27(2), 100338. <https://doi.org/10.1016/j.ctarc.2021.100338>
- Payoungkiattikun, W., Joompang, A., Thongchot, S., Nowichai, B., Jangpromma, N., & Klaynongsruang, S. (2020). Evidence of multi-functional peptide activity: Potential role of KT2 and RT2 for anti-inflammatory, anti-oxidative stress, and anti-apoptosis properties. *Applied Biological Chemistry*, 63(1), 5. <https://doi.org/10.1186/s13765-019-0488-3>.
- Ren, D., Wang, P., Liu, C., Wang, J., Liu, X., Liu, J., & Min, W. (2018). Hazelnut protein-derived peptide LDAPGHR shows anti-inflammatory activity on LPS-induced RAW264.7 macrophage. *Journal of Functional Foods*, 46, 449–455. <https://doi.org/10.1016/j.jff.2018.04.024>
- Sui, H., Wang, F., Weng, Z., Song, H., Fang, Y., Tang, X., & Shen, X. (2020). A wheat germ-derived peptide YDWPGGRN facilitates skin wound-healing processes. *Biochemical and Biophysical Research Communications*, 524(4), 943–950. <https://doi.org/10.1016/j.bbrc.2020.01.162>
- Trott, O., & Olson, A. J. (2009). AutoDock vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, 31(2), 455–461. <https://doi.org/10.1002/jcc.21334>
- Ulug, S. K., Jahandideh, F., & Wu, J. (2021). Novel technologies for the production of bioactive peptides. *Trends in Food Science & Technology*, 108, 27–39. <https://doi.org/10.1016/j.tifs.2020.12.002>
- Vo, T.-S., Ryu, B., & Kim, S.-K. (2013). Purification of novel anti-inflammatory peptides from enzymatic hydrolysate of the edible microalgal *Spirulina maxima*. *Journal of Functional Foods*, 5(3), 1336–1346. <https://doi.org/10.1016/j.jff.2013.05.001>
- Wang, P., Liu, F., Yang, X., Liang, Y., Li, S., Su, G., Jin, D. Q., Ohizumi, Y., Xu, J., & Guo, Y. (2017). Clerodane diterpenoids from *Scutellaria formosana* with inhibitory effects on NO production and interactions with iNOS protein. *Phytochemistry*, 144, 141–150. <https://doi.org/10.1016/j.phytochem.2017.09.005>
- Wang, S., Su, G., Zhang, Q., Zhao, T., Liu, Y., Zheng, L., & Zhao, M. (2018). Walnut (*Juglans regia*) peptides reverse sleep deprivation-induced memory impairment in rat via alleviating oxidative stress. *Journal of Agricultural and Food Chemistry*, 66(40), 10617–10627. <https://doi.org/10.1021/acs.jafc.8b03884>
- Wang, S., Zheng, L., Zhao, T., Zhang, Q., Liu, Y., Sun, B., Su, G., & Zhao, M. (2020). Inhibitory effects of walnut (*Juglans regia*) peptides on neuroinflammation and oxidative stress in lipopolysaccharide-induced cognitive impairment mice. *Journal of Agricultural and Food Chemistry*, 68(8), 2381–2392. <https://doi.org/10.1021/acs.jafc.9b07670>
- Wang, X., Tian, J., Yong, K. T., Zhu, X., Lin, M. C., Jiang, W., Li, J., Huang, Q., & Lin, G. (2016). Immunotoxicity assessment of CdSe/ZnS quantum dots in macrophages, lymphocytes and BALB/c mice. *Journal of Nanobiotechnology*, 14(1), 10. <https://doi.org/10.1186/s12951-016-0162-4>

- Wen, L., Chen, Y., Zhang, L., Yu, H., Xu, Z., You, H., & Cheng, Y. (2016). Rice protein hydrolysates (RPHs) inhibit the LPS-stimulated inflammatory response and phagocytosis in RAW264.7 macrophages by regulating the NF- κ B signaling pathway. *RSC Advances*, 6(75), 71295–71304. <https://doi.org/10.1039/C6RA08927E>
- Xue, L. L., Wu, W. S., Ma, X., Pei, H. Y., Tang, M. H., Kuang, S., Cai, X. Y., Wang, L., Li, Y., Zhang, R. J., Hong, F., Peng, A. H., Ye, H. Y., & Chen, L. J. (2020). Modulation of LPS-induced inflammation in RAW264.7 murine cells by novel isoflavonoids from *milletia pulchra*. *Bioorganic Chemistry*, 97, 103693. <https://doi.org/10.1016/j.bioorg.2020.103693>
- Yi, G., Li, H., Liu, M., Ying, Z., Zhang, J., & Liu, X. (2020). Soybean protein-derived peptides inhibit inflammation in LPS-induced RAW264.7 macrophages via the suppression of TLR4-mediated MAPK-JNK and NF-kappa B activation. *Journal of Food Biochemistry*, 44(8), e13289. <https://doi.org/10.1111/jfbc.13289>
- Zabłocka, A., Siednienko, J., Mitkiewicz, M., Gorczyca, W. A., Lisowski, J., & Janusz, M. (2010). Proline-rich polypeptide complex (PRP) regulates secretion of inflammatory mediators by its effect on NF- κ B activity. *Biomedicine & Pharmacotherapy*, 64(1), 16–20. <https://doi.org/10.1016/j.biopha.2009.01.009>
- Zhang, Q., Lenardo, M. J., & Baltimore, D. (2017). 30 years of NF- κ B: A blossoming of relevance to human pathobiology. *Cell*, 168(1-2), 37–57. <https://doi.org/10.1016/j.cell.2016.12.012>
- Zhang, Q., Su, G., Zhao, T., Sun, B., Zheng, L., & Zhao, M. (2020). Neuroprotection of round scad (*Decapterus maruadsi*) hydrolysate in glutamate-damaged PC12 cells: Possible involved signaling pathways and potential bioactive peptides. *Journal of Functional Foods*, 64, 103690. <https://doi.org/10.1016/j.jff.2019.103690>
- Zinatizadeh, M. R., Schock, B., Chalbatani, G. M., Zarandi, P. K., Jalali, S. A., & Miri, S. R. (2021). The nuclear factor kappa B (NF- κ B) signaling in cancer development and immune diseases. *Genes & Diseases*, 8(3), 287–297. <https://doi.org/10.1016/j.gendis.2020.06.005>