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Research Article

Huarong Large-Leaf Mustard Extracts Exhibit Antioxidant Activity and Inhibition of TLR4-Mediated Inflammatory Response in Murine Macrophage

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Leaf mustard is rich in bioactive components such as polyphenols and glucosinolates, which reportedly have antioxidant and anti-inflammatory activities. Here, in this study, we investigated the potential antioxidant and anti-inflammatory effects of fresh leaf mustard extract (FrLME) and fermented leaf mustard extract (FeLME) using a lipopolysaccharide (LPS)-induced *in vitro* antioxidant model and LPS-induced RAW264.7 inflammation model. The results showed that both FrLME and FeLME exhibit antioxidant activity in ABTS, DPPH, and FRAP assays in linear. In the LPS-induced inflammatory cells, analyses of SOD and GSH-PX activities indicated that the FrLME group exhibited significantly higher activity compared to the FeLME group ($p < 0.05$). A comparative assessment of the extracts indicated that the levels of nitrites (NO) and inflammatory cytokines (TNF-α, IL-1β, and IL-6) were considerably lower in cells treated with FrLME than in those treated with FeLME ($p < 0.05$). Moreover, treatment with FrLME and FeLME effectively mitigated cellular inflammation, as evidenced by the significantly reduced mRNA and protein expression of the TLR4-NF-κB p65-COX-2/iNOS pathway markers during LPS-induced inflammation. Notably, extracts from fresh leaves demonstrated more pronounced inhibitory effects compared to those from fermented leaf mustard. The outcomes of this study establish a foundation for exploring the antioxidant and anti-inflammatory mechanisms of leaf mustard in more intricate models of inflammatory diseases.

1. Introduction

In recent years, several studies have investigated the phytochemical components of leaf mustard (*Brassica juncea*) and their potential functional effects against some inflammatory diseases [1]. Huarong leaf mustard is a new excellent variety bred by researchers in Hunan Province, that is annually grown on over 30,000 ha in Hunan Province, China. However, no reports have yet explored its functional effects or provided *in vitro* evidence of its anti-inflammatory or antioxidant activity.

Inflammation has been well-established to have a close relationship with oxidative stress since high levels of reactive oxygen species (ROS) are produced during an inflammatory response, inducing oxidative stress, which in turn serves as a feedback to promote inflammation [2]. A study by Oh et al. [3] demonstrated that a 50% acetonitrile extract of Korean Dolsan leaf mustard exhibited antioxidant activity in 2,2-azino-bis(3-ethyl-benzothizoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and ferric-reducing antioxidant power (FRAP) assays. This antioxidant activity had a linear correlation with the polyphenol and flavonoid

contents of the extract, leading them to conclude that these effects may be mediated by polyphenols and flavonoids [3]. Similarly, Huang et al. [4] studied the antioxidative potential of ethanol extracts of leaf mustard (*Brassica juncea* Coss. var. *foliosa* Bailey) and found that they showed strong antioxidant activity in increasing the shelf-life of raw meat by decreasing the levels of thiobarbituric acid and free fatty acids [4]. Another study by Young Kim et al. [5] also demonstrated that the leaf mustard (*Brassica juncea*) extract could reduce lipid peroxidation and oxygen-free radical levels and improve the damage due to oxidative stress in rats with streptozotocin-induced diabetes [5]. In addition, other studies have reported that leaf mustard extract provided a significant protective effect against inflammatory response in cells exposed to lipopolysaccharide (LPS), suggesting that LME provides anti-inflammatory properties [6].

Based on initial data showing that extracts of both fresh and fermented leaf mustard (FrLME and FeLME, respectively) are rich in polyphenols, flavonoids, glucosinolates, and other components (unpublished), extracts of Huarong large-leaf mustard may have antioxidant or anti-inflammatory properties. Therefore, to investigate the potential antioxidant and anti-inflammatory effects of FrLME and FeLME, *in vitro*, in this work, we used a lipopolysaccharide (LPS)-induced RAW264.7 murine macrophage model for inflammation and oxidative stress, in addition to other conventional assays for free radical scavenging and antioxidant activity. In particular, we examined the specific effects of FrLME and FeLME against several ABTS, DPPH, and FRAP; studied the activity of antioxidant enzymes SOD, GSH-PX, and MDA content; tested LME toxicity towards cells; measured the contents of several inflammatory cytokines under exposure to LMEs during LPS induction of inflammatory response; and ultimately detected changes in the expression of TLR4 and downstream targets during inflammation. This work provides experimental, *in vitro* evidence of antioxidant and anti-inflammatory signaling activities that can serve as a basis for studies in more sophisticated models of inflammatory disease that urgently need new therapeutic avenues, such as inflammatory bowel disease.

2. Materials and Methods

2.1. Materials and Chemicals. Fresh and fermented Huarong large-leaf mustards were purchased from Huarong County, Hunan Province, China. Both fresh and fermented leaf mustards were freeze-dried and ground into powder and then they were stored at −80°C for later use.

RAW264.7 cell was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). ABTS, DPPH, tri-pyridyl-triazine (TPTZ), iron (III) chloride hexahydrate, streptomycin, penicillin, methyl thiazolyl tetrazolium (MTT), lipopolysaccharide (LPS), trypsin, ascorbic acid, and antiβ-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) was obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone (Mediatech, Herndon, VA). Dimethyl sulfoxide (DMSO), isopropanol, ethanol, and chloroform of molecular biological grade were obtained from Fisher Scientific (Pittsburg, PA, USA). RNase inhibitor, High-Capacity cDNA Reverse Transcription Kit, and Green master mix SYBR were supplied by Fisher Scientific (Pittsburg, PA, USA). TRIzol RNA extractors and primers were purchased from Integrated DNA Technologies (Skokie, IL, USA). Antibody iNOS and cyclooxygenase-2 (COX-2) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibody toll-like receptor 4 (TLR4) and nuclear factor kappa-B (NF-κB p65) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Preparation of the Extract. Extractions were conducted in accordance with a previous study with some modifications [7]. Fresh and fermented leaf mustard powder were separately blended with 70% ethanol solution (v/v) in a ratio of 1 : 25 (w/w), extracted in a constant temperature water bath at 70°C for 30 min, and then centrifuged at 4000 × g for 10 min (Thermo Scientific, Waltham, MA, USA). The supernatant was concentrated to a certain volume with a rotary evaporator and then the fresh leaf mustard extract was vacuum freeze-dried to obtain FrLME.

In addition, the fermented leaf mustard extract solution was desalted as follows: the extract solution was put into a dialysis bag, sealed, and put into distilled water for dialysis. The dialysate was regularly replaced until sodium chloride could not be detected and then the dialysate was concentrated and freeze-dried to obtain FeLME.

2.2.1. ABTS-Free Radical Scavenging Method. ABTS radical scavenging activity was measured according to a previous study with minor modification [3]. In brief, 5 mL of ABTS solution (7.4 mmol/L) and 88 μL of potassium peroxodisulfate (2.6 mmol/L) were mixed and reacted for 12 hours to obtain ABTS radical solution. ABTS radical solution (5 mL) was added to 0.1 mL samples of different concentrations (50, 100, 150, 200, 300, 400, 500, and 600 μg/μL leaf mustard extract) and reacted in the dark for 6 min. The absorbance was measured at 734 nm (indicated by A1) and repeated three times. The sample blank was used as a control (indicated by A0). ABTS radical scavenging activity (%) = (1 − A1/A0) × 100. Ascorbic acid was used as a positive control.

2.3. DPPH Radical Scavenging Method. DPPH radical scavenging ability was measured according to a previous study with minor modification [3]. In brief, 0.1 mmol/L of DPPH solution was prepared with absolute ethanol, 2 mL of DPPH solution was mixed with 2 mL of different sample concentrations (50, 100, 150, 200, 300, 400, 500, 600 μg/μL leaf mustard extract), and they reacted in the dark at room temperature for 30 minutes. The absorbance was measured at 520 nm (indicated by A1), repeated three times, and the sample blank was used as a control (indicated by A0). DPPH radical scavenging ability (%) = (1 − A1/A0) × 100. Ascorbic acid was used as a positive control.

2.3.1. FRAP Ferric Ion-Reducing Antioxidant Power Measurement. Ferric ion-reducing antioxidant power (FRAP) was measured according to a previous study with a minor modification [3]. In brief, acetate buffer (30 mM, pH 3.6), TPTZ (10 mM), and iron (III) chloride hexahydrate (20 mM) were mixed evenly in a 10 : 1:1 ratio to obtain a reaction mixture. A total of 2.4 mL reaction mixture was added to 0.1 mL samples of different concentrations (0, 50, 100, 150, 200, 300, 400, 500, and 600 μg/μL leaf mustard extract). The absorbance was measured at 593 nm and repeated three times. FRAP activity (mg FeSO4 equivalents/g) was calculated according to the iron (II) sulfate heptahydrate standard curve. Ascorbic acid was used as a positive control.

2.3.2. Cell Culture and Treatments. RAW264.7 cells were cultured in the DMEM cell culture medium (1% penicillin-streptomycin double antibody solution and 10% FBS) and incubated in a humidified incubator at 37°C and 5% CO_2, until the cells grew to 80% of the culture dish. The cells were then gently scraped and used to seed the cells in a 96-well plate (200 μL/well, 50 /w10^4 cells/mL); these cells were treated with DMEM (LPS solution (1 mg/ml)) for 24 h to prepare an inflammatory cell model. After 24 hours, the supernatant medium was discarded. DMSO was used as the vehicle to deliver FrLME and FeLME, and the final concentrations were 0, 50, 100, 150, 200, 300, 400, 500, or 600 μg/μL of leaf mustard extract culture in cell culture media, and the control group was RAW264.7 cells without the LPS medium.

2.4. Determination of SOD, MDA, and GSH-PX in Cell Lysate. Preparation of cell lysate: trypsin was added for digestion and the cells were suspended in PBS and centrifuged (4°C, 13,200 × g, 10 min) to obtain a precipitate. Then, 1 mL 1% Triton X-100 was added, centrifuged (4°C, 7,500 × g, 10 min) again, and the supernatant was collected. Superoxide dismutase (SOD) was detected by spectrophotometry, and the absorbance was determined at a wavelength of 550 nm. The detection steps were strictly in accordance with the instructions of the SOD kit (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China). The thiobarbituric acid (TBA) method was used to determine the malondialdehyde (MDA) content of the cell lysate. Absorbance was measured at 532 nm, and the MDA content was calculated according to the standard curve, and the detection steps strictly followed the MDA kit instructions (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China). Glutathione peroxidase (GSH-PX) was detected by spectrophotometry, and the absorbance was measured at a wavelength of 412 nm. The detection steps were strictly in accordance with the instructions of the GSH-PX kit (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China).

2.5. MTT Method to Measure Cell Viability. The RAW264.7 cells were activated and passaged until stabilization. After passaging, they were cultured in a CO_2 incubator at 37°C with 5% humidity. After 24 hours, the cells were seeded onto 96-well plates at a density of 1.25×10^4 cells/mL. The medium was discarded after an additional 24-hour incubation. The freeze-dried powders of FrLME and FeLME were dissolved in DMSO to prepare solutions of the desired concentrations. 3 μL of the DMSO sample solution was then added to 3 mL of the culture medium to obtain final concentrations ranging from 0 to 600 μg/μL of the mustard ethanol extract. Using a pipette, these varying concentrations of mustard ethanol extract solutions were transferred into the 96-well plates (200 μL/well) from which the supernatant had been discarded. After incubation periods of 24 h, 48 h, and 72 h, the inhibitory effects of the mustard ethanol extract on cell proliferation were determined using the MTT assay. The culture medium containing different concentrations of the ethanol extract was discarded, and 100 μL of MTT solution (0.5 mg/mL) was added to each well. After a 2-hour incubation, the MTT solution was gently poured out and replaced with 100 μL of DMSO to dissolve the purple formazan crystals in the wells. The plates were gently tapped on the sides to ensure mixing of the crystals, and the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The cell inhibition rate was then calculated.

2.6. NO Measurement. A pipette was used to remove 150 μL of cell supernatant from each well of the plate, and 100 μL of Griess A and B equal volume mixed solution was added to each well. The absorbance was measured with a microplate reader at 570 nm, and the NO concentration was calculated according to the sodium nitrite ($NaNO_2$) standard curve.

2.7. Determination of Cytokines TNF-α, IL-1β, and IL-6 in the Cell Culture Medium. The detection was performed according to the instructions of the tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) kits (Cloud clone, Houston, CCC, USA).

2.8. Western Blot (WB). WB was conducted as previously reported [8, 9]. The protein content was detected using the Coomassie brilliant blue method; the protein was denatured by heating at 95°C for 10 minutes and then stored in the refrigerator at −80°C for later use. The RAW264.7 cells were seeded in a 25 cm culture dish (cell density of 3.5×10^4 cells/mL, 12 mL). After 24 hours of incubation, the supernatant culture medium was discarded. Each dish was then treated with 12 mL of the mustard ethanol extract culture medium and incubated for 48 hours. Cellular proteins were extracted using lysis buffer (2 × radio-immunoprecipitation assay buffer, RIPA buffer : 100 × protease inhibitor : 100 × phosphatase inhibitor I : 100 × phosphatase inhibitor I in a ratio of 50 : 1 : 1 : 1 v/v). The protein content was measured by Coomassie brilliant blue staining. The sample was then mixed with an appropriate volume of 5 × protein loading buffer, denatured at 95°C for 10 minutes, and stored at −80°C for future use. A 12% acrylamide separating gel (50 mL) was prepared using the following components: 16.5 mL of distilled water, 20 mL of 30% acrylamide solution

(m/v), 12.5 mL of Tris solution (1.5 mol/L, pH 8.8), 0.5 mL of 10% SDS (m/v), 0.5 mL of 10% ammonium persulfate (m/v), and 0.02 mL of TEMED. Approximately 6 mL of this mixture was pipetted into a gel casting mold and allowed to polymerize for 30 minutes. A 5% acrylamide stacking gel (50 mL) was prepared using 34 mL of distilled water, 8.5 mL of 30% acrylamide solution (m/v), 6.25 mL of Tris solution (1.0 mol/L, pH 6.8), 0.5 mL of 10% SDS (m/v), 0.5 mL of 10% ammonium persulfate (m/v), and 0.02 mL of TEMED. About 2 mL of this mixture was pipetted into a gel casting mold and polymerized for 60 minutes. Both stacking and separating gels were mounted onto the electrophoresis apparatus. Samples (5 μL each) and protein markers (5 μL) were loaded onto the gel. Electrophoresis was performed at 100 V for 15 minutes. Once the samples were about to migrate from the stacking gel to the separating gel, the voltage was increased to 160 V and continued for another 45 minutes or until the target proteins were completely separated. The gel was then subjected to protein transfer at 100 V for 1 hour and 45 minutes. The membrane was blocked, incubated with primary and secondary antibodies, and finally imaged using an infrared imaging system.

2.9. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis. qRT-PCR was conducted as previously reported [10]. The primer pairs were synthesized by Integrated DNA Technologies (Skokie, IL, USA). Total RNA extraction: RAW264.7 cells were seeded in a 25 cm culture dish (cell concentration of 3×10^5 cells/mL, 12 mL) and cultured for 24 h. The supernatant was then discarded, and 12 mL of the mustard ethanol extract was added to each dish and incubated for another 48 h. After discarding the supernatant again, 0.5 mL of the TRIzol reagent was added and mixed. The cells were left to stand for 5 min to ensure complete dissolution. Subsequently, 0.1 mL of chloroform was added, shaken well, and lightly vortexed before allowing to stand for 3 min. Centrifugation was carried out (12,000 r/min, 4°C, 15 min), and the supernatant was collected and mixed with 0.25 mL of isopropanol. After shaking and standing for 10 min, another centrifugation step was performed (12,000 × g, 4°C, 10 min). The supernatant was discarded, leaving the pellet, to which 0.5 mL of 75% ethanol was added and mixed. Following another centrifugation (7,500 × g, 4°C, 5 min), the supernatant was removed, and the pellet was vacuum-dried for 5 min. The RNA was then dissolved in 30 μL of RNase-free water. The RNA concentration was measured at 280 nm UV, and aliquots were stored at −80°C for future use. Reaction conditions: the cDNA synthesis was performed as per the protocol provided by the Applied Biosystems reverse transcription kit. The cDNA reaction parameters were set at 25°C for 10 min, followed by 37°C for 120 min, and finally, 85°C for 5 min. The qRT-PCR cycling parameters were 95°C for 10 min of initial denaturation, followed by 95°C for 120 s, 95°C for 15 s, 50°C for 120 s, and 60°C for 60 s for a total of 40 cycles. GADPH was used as an endogenous control, and the samples were normalized based on GADPH content. The relative quantification of the target gene mRNA expression levels was calculated using the $2^{-\Delta\Delta Ct}$ method, and each sample was tested in three independent replicates.

2.10. Statistical Analysis. Image J software was used to process the optical density of the WB target protein band for quantification. Statistical evaluations were performed using SPSS 25.0 (Chicago, IL, USA) and GraphPad Prism 8 software (La Jolla, CA, USA). Data are displayed as the mean ± standard deviation (SD). Group differences were assessed via one-way ANOVA, succeeded by Tukey's post hoc analysis. For comparing the two groups, a two-tailed Student's *t*-test was applied. $p < 0.05$ indicated that the difference was statistically significant.

3. Results

3.1. Both Fresh and Fermented Leaf Mustard Extracts Exhibit Antioxidant Activity. Excessive free radicals produced by metabolic processes under stress conditions can damage lipids and proteins that comprise cellular structures, as well as DNA, through peroxidation, causing oxidative damage [11, 12]. In order to determine the ability of leaf mustard extracts to eliminate excessive free radicals and thus prevent oxidative damage, we used DPPH and ABTS to measure the free radical scavenging and FRAP to evaluate the antioxidant activity by fresh and fermented leaf mustard extracts. We found that ABTS radical scavenging activity gradually increased as the concentration of the leaf mustard extract increased (Figure 1(a)). At a concentration of 300 μg/μL, the ABTS radical scavenging activities for FrLME were 73% and 62% for FeLME. Similarly, DPPH radical scavenging ability gradually increased along with the concentration of either LME (Figure 1(b)), exhibiting maximum scavenging at 400 μg/μL. At this concentration, FrLME scavenged 36% of the DPPH radicals, while FeLME neutralized 31%. Analysis of FRAP by leaf mustard extracts also showed that antioxidant activity increased along with concentration for both extracts, up to 400 μg/μL (Figure 1(c)). At this concentration, FrLME and FeLME exhibited FRAP activities of 136 and 115 mg $FeSO_4$ equivalents/g, respectively. As with free-radical scavenging, their activity did not significantly change with the addition of higher extract concentrations. Taken together, these results indicate that both FrLME and FeLME showed antioxidant activity in a concentration-dependent manner up to 400 μg/μL ($p < 0.05$). The effect of FrLME and FeLME (200–600 μg/μL) in scavenging free radicals was equivalent to that of ascorbic acid (1 μg/μL). These abovementioned results demonstrated that both FrLME and FeLME could effectively scavenge free radicals.

3.2. Leaf Mustard Extracts Rescue SOD and GSH-PX Activity and MDA Content during Inflammation in RAW264.7 Cells. SOD and GSH-PX actively scavenge free radicals during inflammation, and MDA levels can serve as a marker for antioxidant capacity, reflecting the potential rate and intensity of lipid peroxidation in the body (or as an indirect measure of damage by tissue peroxidation) [13, 14]; we, therefore, analyzed the effects of FrLME and FeLME on these enzymes by spectrophotometry in RAW264.7 cell lysates. We found that compared with the no LPS controls,

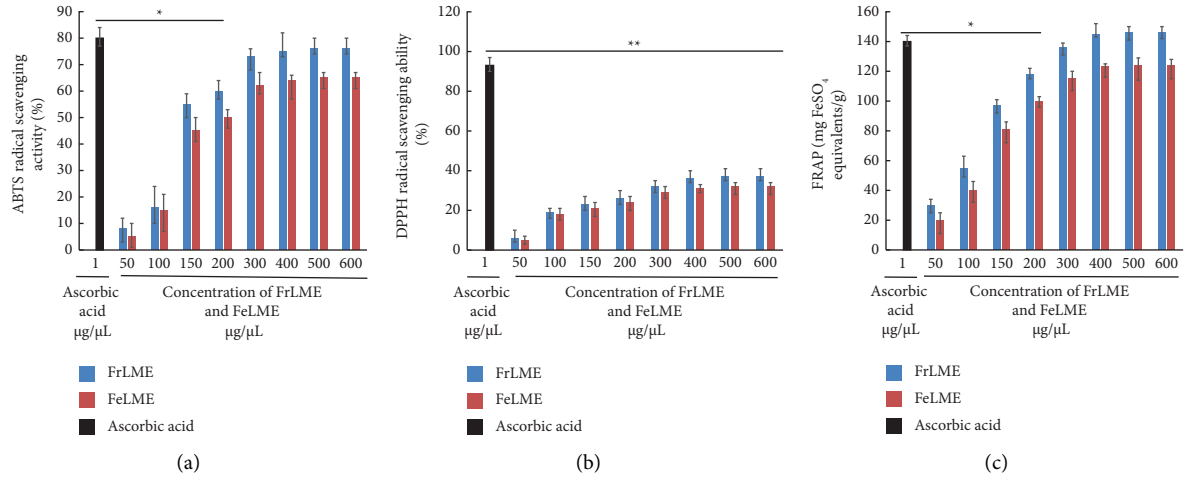

Figure 1: Antioxidant effects of FrLME and FeLME on (a) ABTS, (b) DPPH, and (c) FRAP. Ascorbic acids were used as positive controls in (a–c), respectively. All data are expressed as the mean ± SD ($n = 3$) and are representatives of three separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. positive control.

exposure to LPS significantly reduced SOD and GSH activity ($p < 0.05$), whereas treatment with leaf mustard extracts in these LPS-stimulated inflammatory response cells resulted in significantly higher SOD (Figure 2(a)) and GSH-PX (Figure 2(b)) activity at both 150 and 300 μg/μL extract concentrations. By contrast, LPS stimulation significantly increased the content of MDA in RAW264.7 cell lysate ($p < 0.05$), whereas the MDA content was significantly lower under exposure to 150 μg/μL and 300 μg/μL of either LMEs in LPS-induced treated inflammatory cells (Figure 2(c)).

Comparison of differences in the effects FrLME and FeLME on SOD and GSH-PX activities showed that the FrLME group exhibited significantly higher activity than that of the FeLME group, although MDA levels were not significantly different between the extract treatments ($p < 0.05$). Overall, these results suggested that both extracts could effectively inhibit the inflammation-associated decrease in SOD and GSH-PX antioxidant activity and increased MDA content in a dose-dependent manner in LPS-induced RAW264.7 inflammatory cells.

3.3. Treatment with Leaf Mustard Extracts Results in Lower NO Accumulation in LPS-Induced Inflammatory Cells. Given the observed antioxidant and anti-inflammatory properties, we further explored the potential impacts of FrLME and FeLME on cell viability. We selected specific concentrations based on prior research and aimed to discern their efficacy using MTT assays. The results showed that compared with untreated control cells, exposure to either extract at concentrations up to 600 μg/μL resulted in no significant decrease in cell viability after 24 h incubation (Figure 3(a)). Moreover, no significant differences were observed in cell viability between the FrLME and FeLME groups. These results showed that neither leaf mustard extract produced toxic effects on RAW264.7 cells.

Since the NO content is a reliable indicator of inflammation, we then examined whether exposure to either LME resulted in changes to the NO content in RAW264.7 cells under inflammatory conditions. The results showed that compared with the uninduced control group, the NO content was significantly elevated in LPS-stimulated cells ($p < 0.001$) (Figure 3(b)). By contrast with LPS-induced cells, increasing concentrations of FrLME and FeLME from 50 to 400 μg/μL led to significantly reduced NO content, in a concentration-dependent manner ($p < 0.01$). Concentrations higher than 400 μg/μL did not result in a further reduction in the NO content. In addition, comparison between FrLME and FeLME treatments showed that the NO content was significantly lower in cells exposed to FrLME ($p < 0.05$). These results indicated that exposure to both FeLME and FrLME led to the effective inhibition of NO accumulation associated with LPS-induced inflammation in RAW264.7 cells.

3.4. Treatment with Leaf Mustard Extracts Results in Lower Inflammatory Factor Secretion by LPS-Induced RAW264.7 Cells. TNF-α, IL-1β, and IL-6 are recognized as pivotal proinflammatory factors. To understand the inflammation-associated pathways influenced by FrLME and FeLME, we used ELISA-based assays to evaluate the levels of these inflammatory markers. The results showed that compared with uninduced controls, LPS stimulation significantly increased the levels of the cytokines TNF-α, IL-1β, and IL-6 secreted by RAW264.7 cells ($p < 0.05$). In contrast, TNF-α (Figure 4(a)), IL-1β (Figure 4(b)), and IL-6 (Figure 4(c)) levels were significantly reduced ($p < 0.05$) in an apparently concentration-dependent manner, following treatment with 150 and 300 μg/μL concentrations of either extract in LPS-induced inflammatory cells. Comparison between extracts showed that all three cytokine markers were significantly lower in cells treated with FrLME than in those treated with

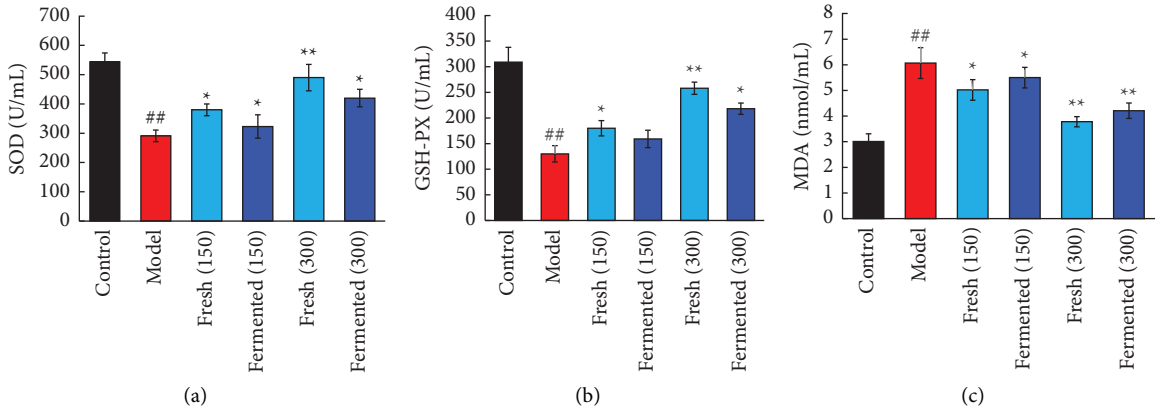

Figure 2: Effects of the mustard extract on (a) SOD, (b) GSH-PX, and (c) MDA in LPS-treated RAW264.7 cells. Fresh (100) represents FrLME 100 $\mu g/\mu L$, fresh (150) represents FrLME 150 $\mu g/\mu L$, fermented (100) represents FeLME 100 $\mu g/\mu L$, and fermented (150) represents FeLME 150 $\mu g/\mu L$. Data are expressed as the mean ± SD ($n = 4$–7). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. control. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. model.

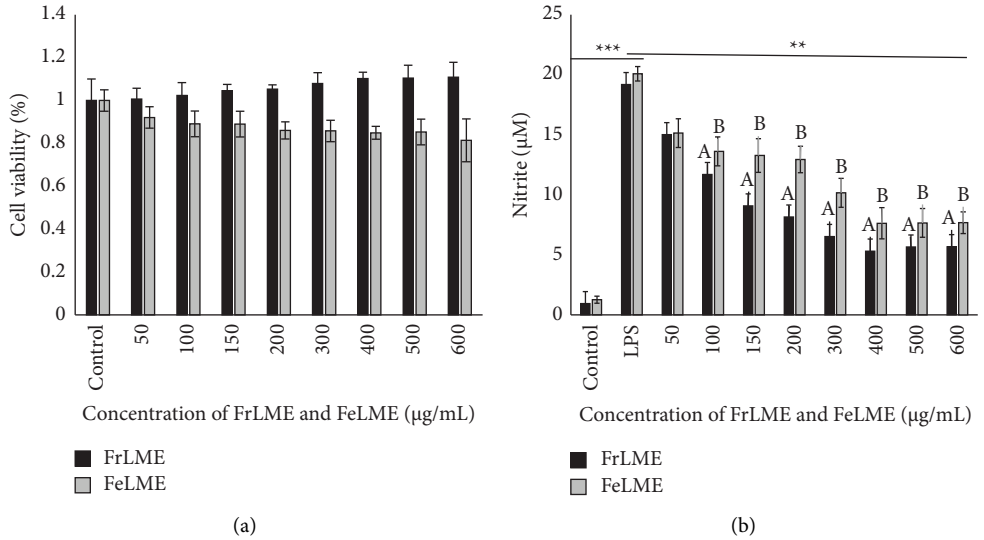

Figure 3: The effects of FrLME and FeLME on RAW264.7 cell viability and NO. (a-b) The results of cell viability and NO content of RAW264.7 cells treated with two extracts at different concentrations (0–600 $\mu g/\mu L$) for 24 h, respectively. The data are represented as the mean ± SD ($n = 6$); A and B indicate that there was a significant difference between the average values of the two groups of data ($p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

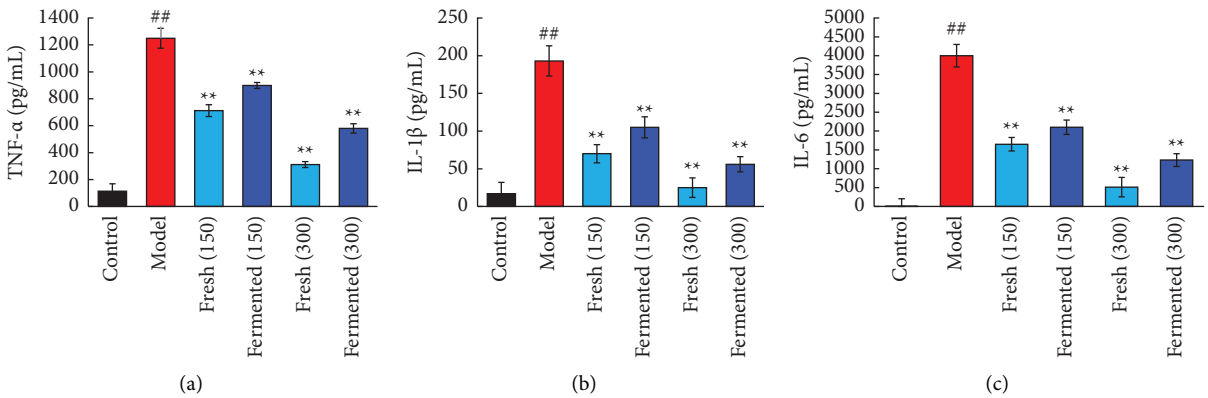

Figure 4: Effects of FrLME and FeLME on (a) TNF-α, (b) IL-1β, and (c) IL-6 levels in LPS-treated RAW264.7 cells. Data are expressed as the mean ± SD ($n = 4$–7). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. control. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. model.

FeLME ($p < 0.05$). This decrease in inflammatory factors in LPS-induced RAW264.7 cells suggested that exposure to fresh and fermented LMEs could produce significant inhibitory effects on the inflammatory pathway.

3.5. Effect of the Leaf Mustard Extract on RAW264.7 Cell Inflammation Signal Pathway-Related Genes and Proteins. The TLR4/NF-κB signaling pathway is the "star pathway," involved in intestinal immune inflammatory response [15]. In order to explore the potential mechanisms by which leaf mustard extracts affect the inflammatory response, we used qRT-PCR and Western blots to, respectively, examine the transcription and protein expression of TLR4 and its downstream targets NF-κB p65, COX-2, and iNOS in RAW264.7 cells. These assays revealed that in comparison with the uninduced control group, the mRNA levels of TLR4 (Figure 5(a)), NF-κB p65 (Figure 5(b)), COX-2 (Figure 5(c)), and iNOS (Figure 5(d)) increased significantly ($p < 0.05$) by 2-fold, 0.82-fold, 0.7-fold, and 5.5-fold, respectively, under exposure to LPS. Consistent with our previous observations of leaf mustard extracts, TLR4, NF-κB p65, COX-2, and iNOS transcription levels were partially restored to that of uninduced cells by FrLME and FeLME treatments at 150 and 300 μg/μL.

Compared with the LPS-induced cells, mRNA expression of TLR4, NF-κB p65, COX-2, and iNOS was, respectively, downregulated by 56.6%, 34.1%, 23.5%, and 36.9% under treatment with 150 μg/μL FrLME and 63.3%, 56%, 32.4%, and 56.5%, respectively, by 300 μg/μL FrLME. Similarly, treatment with 150 μg/μL FeLME led to 30%, 19.2%, 14.7%, and 23.1% reductions in TLR4, NF-κB p65, COX-2, and iNOS mRNA expression, respectively, while greater reductions of 40%, 25.8%, 23.5%, and 38.5% were, respectively, observed at 300 μg/μL FeLME. As in other experiments, exposure to FrLME produced stronger effects than FeLME in induced RAW264.7 cells.

Consistent with our observations of mRNA expression, exposure to LPS significantly increased protein levels of TLR4, NF-κB p65, COX-2, and iNOS ($p < 0.05$) by 0.1-fold, 1.6-fold, 5.4-fold, and 1.7-fold, respectively. Treatment with FrLME or FeLME partially rescued TLR4, NF-κB p65, COX-2, and iNOS protein levels to that of uninduced controls (Figure 5(e)). Compared with the LPS-induced cells, Western blot analysis showed that TLR4, NF-κB p65, COX-2, and iNOS protein levels were decreased by 27.3%, 53.8%, 32.8%, and 14.8% under 150 μg/μL FrLME and 36.4%, 88.5%, 65.6%, and 25.9%, respectively, under 300 μg/μL FrLME. At 150 μg/μL FeLME, TLR4, NF-κB p65, COX-2, and iNOS proteins were reduced by 9.1%, 46.2%, 18.75%, and 7.4% compared to LPS-only treated cells and reduced to a greater extent by 18.2%, 61.5%, 48.4%, and 22.2%, respectively, under 300 μg/μL FeLME treatment. In summary, exposure to either FrLME or FeLME led to significant attenuation in the expression of TLR4 pathway-associated proteins.

4. Discussion

In this work, we examined the antioxidant and anti-inflammatory effects of fresh and fermented leaf mustard extracts, which are commonly eaten for these purposes based on traditional medicines, but which have not been widely investigated for their effects in controlled *in vitro* experiments. Leaf mustard was previously reported to contain high levels of vitamin A, vitamin C, β-carotene, flavonoids, and glucosinolates, etc., and provide antioxidant effects through consumption [1, 16, 17]. Our previous findings have shown that that FrLME and FeLME are both rich in bioactive components, especially polyphenols, glucosinolates, and their derivatives, and that, notably, FrLME has almost double the glucosinolate content of FeLME [18]. In light of other published findings that showed that several glucosinolates can exhibit anticancer, antioxidant, anti-inflammatory, and antibacterial effects [1, 19, 20], it is possible that these sulfur-containing glucosinolate metabolites may contribute to the anti-inflammatory effects observed here, and moreover, their higher contents in FrLME may be related to its overall higher antioxidant activity compared to FeLME.

In this study, we explored the antioxidant and anti-inflammatory properties of extracts from fresh and fermented leaf mustard. While these extracts are traditionally consumed for their therapeutic benefits, systematic *in vitro* investigations into their effects have been limited. Our previous experiments have shown that both FrLME and FeLME are abundant in bioactive compounds, notably polyphenols, glucosinolates, and their derivatives. Intriguingly, FrLME possesses nearly twice the glucosinolate content compared to FeLME. Considering published reports that highlight the potential of various glucosinolates in exhibiting anticancer, antioxidant, anti-inflammatory, and antibacterial activities [1, 19, 20], it is plausible that these sulfur-rich glucosinolate metabolites play a role in the observed anti-inflammatory effects. In addition, the higher glucosinolate content in FrLME could explain its superior antioxidant activity compared to FeLME.

Our study provides experimental evidence that both FrLME and FeLME can scavenge DPPH- and ABTS-free radicals and reduce oxidative ferric ions to block their oxidative effects, in addition to partially restoring activities of SOD and GSH-PX and MDA content in RAW264.7 cells. In an endotoxin-induced oxidative stress model in mice, SOD was shown to ameliorate inflammation and lung tissue damage by stabilizing vascular endothelial function and reducing the levels of vascular endothelial adhesion factors [21]. MDA is a biomarker for peroxidative damage to lipids in the endoplasmic reticulum membrane [22]. GSH-PX is an enzymatic inhibitor of free radicals that inhibits the peroxidation of membrane lipids and thus performs essential functions in regulating oxidation and antioxidant balance in the body [23]. When comparing the effects of FrLME and FeLME on SOD and GSH-PX activities, the FrLME group exhibited significantly greater activity than the FeLME group.

Since oxidative stress is closely related to inflammation due to the massive production of ROS during inflammatory response, ROS promotes the activation of the NF-κB and COX2 transcription factors, resulting in an inflammation signal cascade [24]. We also evaluated the effects of LMEs on inflammation signaling biomarkers in RAW264.7 macrophages. We found that treatment with both FrLME and

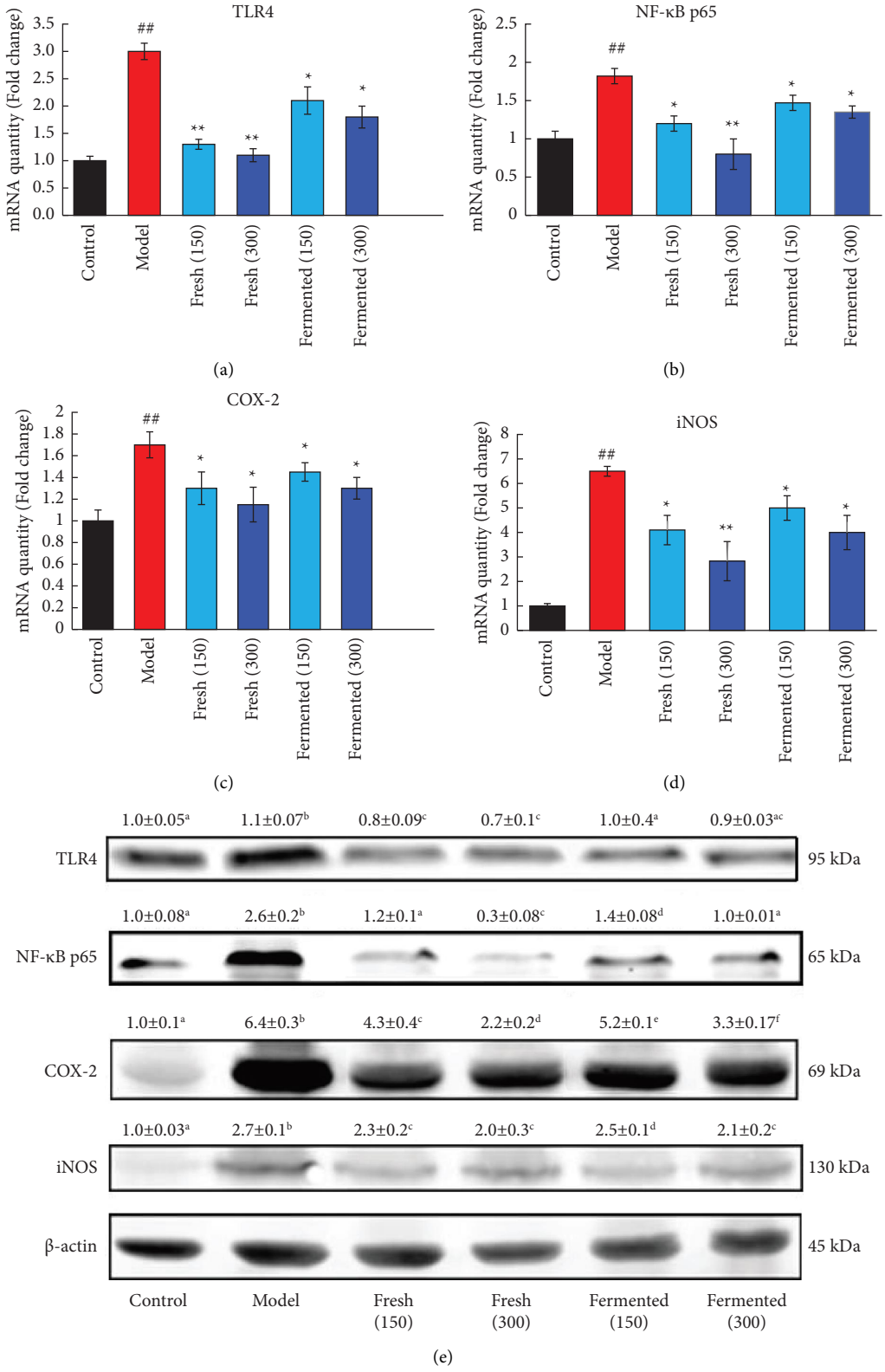

Figure 5: The effect of FrLME and FeLME on inflammation-related mRNA (a–d) and protein (e) of RAW264.7 cells. The data are represented as mean ± SD ($n = 3$). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs control. $^{*}P < 0.05$; $^{**}P < 0.01$ vs model, and the difference between letters indicates a statistically significant difference ($P < 0.05$).

FeLME can lead to significant, concentration-dependent reduction in the secretion of NO by inflammatory cells, the high production of which reportedly leads to aggravation of inflammatory bowel disease via inhibition of colon movement and damage to the intestinal mucosal barrier [25]. Specifically, nitric oxide (NO) is known to increase the permeability of microvascular walls, a process that can be influenced by IL-2 activation. This increase in permeability can lead to dysfunction in the colon, causing tissue congestion and inflammation [25]. In addition to NO, TNF-α, IL-1β, and IL-6 are major proinflammatory factors in ulcerative colitis which are significantly increased in animal models of colitis [26]. Our results showed that treatment with fresh or fermented LME can lead to significantly lower levels of TNF-α, IL-1β, and IL-6 secreted by inflammatory cells, suggesting that these extracts have an inhibitory effect on the production of inflammatory factors. The comparison between FrLME and FeLME treatments shows that the NO content and the levels of TNF-α, IL-1β, and IL-6 in cells treated with FrLME are significantly reduced ($p < 0.05$).

In order to refine our understanding of the mechanisms by which LMEs can induce anti-inflammatory effects in cells, we also examined the mRNA and protein expression of TLR4 and its downstream targets NF-κB p65, COX-2, and iNOS in RAW264.7 cells. Notably, TLR4, NF-κB, COX-2, and iNOS are typically expressed at very low levels in normal human colon tissues but their expression is significantly increased in patients with inflammatory bowel disease [27–30], an effect that we recapitulated in the LPS-stimulated RAW264.7 murine macrophage cells and which was significantly decreased under treatment with both FrLME and FeLME. The TLR4-NF-κB p65-COX-2/iNOS signaling pathway is a classic inflammation signaling pathway, and based on our findings, we hypothesized that the intervention of LME, particularly FrLME, significantly induced anti-inflammatory outcomes by inhibiting this pathway amidst inflammation. We found that both COX-2, a reliable biomarker for enzymatic conversion of arachidonic acid to prostaglandins during oxidative stress [31, 32], and iNOS, which directly increases NO production from L-arginine during inflammation [33] were upregulated by LPS but decreased under LME treatment. Our future experiments will explore the mechanistic interactions by which LME and its specific components block the upregulation of TLR4 signaling through antioxidant activity. In addition, our previous research results indicate that the total glucosinolate (GSL) content is high in the ethanol extracts of FrLME and FeLME, with the total GSL content in FrLME being significantly higher than that in FeLME. In addition, we identified various GSL components in the ethanol extract, including radish glucosinolate (4-methylsulfinylbutyl GSL) and allyl GSL. Therefore, we speculate that the anti-inflammatory activity of the mustard ethanol extract is related to its GSL and its degradation components. Concurrently, the mustard ethanol extract has the potential to become a functional food ingredient for the prevention of inflammatory bowel diseases.

5. Conclusion

In summary, FrLME and FeLME can eliminate DPPH- and ABTS-free radicals and reduce oxidative ferric ions *in vitro*. In the LPS-induced inflammatory RAW264.7 cells, treatment with LMEs can enhance the activity of antioxidant enzymes (i.e., SOD and GSH-PX) and MDA content and lead to reduction in the levels of proinflammatory factors TNF-α, IL-1β, and IL-6. Both extracts exhibited anti-inflammatory effects through apparent blockade of the TLR4-NF-κB p65-COX-2/iNOS signaling pathway. Moreover, FrLME induced stronger anti-inflammatory effects than FeLME, which we speculate may be attributable to the higher glucosinolate content in fresh leaf mustard. Although we provide *in vitro* experimental evidence that mustard extract has anti-inflammatory and antioxidant properties, the basis of material action of the mustard extract has not been fully revealed. In addition, the metabolic pathway and mechanism of the mustard extract *in vivo* still need to be further studied. Upcoming research will delve deeper into sophisticated in *vivo* models to ascertain the potential benefits of these extracts, sourced from both fresh and fermented leaf mustard. We aim to explore their potential anti-inflammatory impacts on conditions such as inflammatory bowel disease, cardiovascular disease, rheumatoid arthritis, and cancer, building upon the antioxidant activity we noted in our in *vitro* observations.

Abbreviations

FrLME: Fresh leaf mustard extract
FeLME: Fermented leaf mustard extract
LME: Leaf mustard extract
LPS: Lipopolysaccharide
ROS: Reactive oxygen species
ABTS: 2,2-Azino-bis(3-ethyl-benzothizoline-6-sulfonic acid)
DPPH: 1,1-Diphenyl-2-picrylhydrazyl
FRAP: Ferric-reducing antioxidant power
Inos: Inducible nitric oxide synthase
PGE2: Prostaglandin E2
TPTZ: Tri-pyridyl-tria-zine
MTT: Methyl thiazolyl tetrazolium
DMEM: Dulbecco's modified Eagle medium
FBS: Fetal bovine serum
DMSO: Dimethyl sulfoxide
COX-2: Cyclooxygenase-2
TLR4: Toll-like receptor 4
NF-κB p65: Nuclear factor kappa-B
SOD: Superoxide dismutase
TBA: Thiobarbituric acid
MDA: Malondialdehyde
GSH-PX: Glutathione peroxidase
$NaNO_2$: Sodium nitrite
TNF-α: Tumor necrosis factor
IL-1β: Interleukin-1β
IL-6: Interleukin-6
WB: Western blot
qRT-PCR:

Quantitative real-time polymerase chain reaction
Mrna: Messenger RNA.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Additional Points

Practical Applications. In recent years, the research on the phytochemical components of leaf mustard and its functional effects, e.g., anticancer, anti-inflammation, and antioxidation, had attracted great attention all over the world. This study reveals the potential applications of leaf mustard extracts in antioxidation and anti-inflammation. Fresh leaf mustard extract (FrLME) and fermented leaf mustard extract (FeLME) demonstrated significant antioxidative activities in various assays, with FrLME showing stronger anti-inflammatory effects in an LPS-induced inflammation cell model. These findings provide a theoretical basis for the application of leaf mustard extracts as natural antioxidants and anti-inflammatory agents in the food industry and pharmaceutical development. Particularly, FrLME exhibited potential in treating inflammation-related diseases by inhibiting the TLR4-NF-κB p65-COX-2/iNOS pathway and reducing the expression of inflammatory factors. Moreover, these results lay the foundation for further research and application of leaf mustard extracts in more complex models of inflammatory diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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