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Research Article

Camellia japonica L. Seed Cake Protects Inflammatory Response in Lipopolysaccharide-Induced Macrophages and Mice

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Camellia japonica L. seed oil has been widely studied and utilized for its skincare, antioxidant, and anti-inflammatory effects. However, the anti-inflammatory activity of the seed cake—a by-product obtained after oil extraction from *C. japonica* L. seeds—has not yet been investigated. This study explored the anti-inflammatory properties and underlying mechanisms of the butanol fraction derived from defatted *C. japonica* L. seed cake (BFCJ). To induce inflammation, RAW 264.7 macrophages were treated and mice were injected with lipopolysaccharide (LPS). The effects of BFCJ on inflammation were measured using nitric oxide (NO) production, ELISA, histopathological analysis, western blotting, serum biochemical analysis, and PCR. Ultra-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC-QTOF MS/MS) was used to characterize the constituents of BFCJ. BFCJ treatment at 5–50 μg/mL dose-dependently decreased NO production and inflammatory cytokine levels including tumor necrosis factor-α, interleukin (IL)-6, and IL-1β in RAW 264.7 cells induced by LPS. BFCJ also downregulated the levels of nuclear factor kappa B (NF-κB) pathway-related proteins, such as phosphorylated NF-κB, inducible nitric oxide synthase, and cyclooxygenase-2. Furthermore, administration of 50 mg/kg/day BFCJ for 14 days attenuated hepatotoxicity, as observed in the histopathological analysis. Compared to LPS treatment in mice, BFCJ significantly lowered inflammatory cytokine and protein levels during NF-κB signaling. Furthermore, UPLC-QTOF MS/MS analysis identified four flavonoids, including kaempferol glycosides, in BFCJ. This study suggests that BFCJ, which contains flavonoids, is a potential anti-inflammatory agent. Identifying and characterizing the functional properties of this seed cake will provide a scientific basis for its development and application as a functional ingredient in food and pharmaceutical products.

Keywords: *Camellia japonica* L.; inflammation; lipopolysaccharide; nitric oxide; seed cake

1. Introduction

Inflammation is an immune response to external stimuli through various reactions in the body, including the release of nitric oxide (NO) and inflammatory cytokines by macrophages [1]. However, an excessive inflammatory response is associated with the prevalence of diabetes, cancer, and other diseases [2]. A nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) signaling mediates major inflammatory responses [3]. Phosphorylated NF-κB (pNF-κB) enhances the expression of inflammatory mediators such as inducible nitric oxide synthase (iNOS) and

cyclooxygenase-2 (COX-2), leading to the overgeneration of inflammatory cytokines and ultimately causing cellular or tissue damage in the body [3, 4]. Therefore, research on anti-inflammatory strategies targeting inhibition of NF-κB signaling pathway activation is critically necessary; hence, many studies have been reported in the field of phytotherapy and phytopharmacology [5].

Camellia japonica L., a flowering plant in *Theaceae*, originates from East Asia and is distributed across tropical and subtropical regions worldwide [6]. *C. japonica* L. is traditionally used for various purposes, including tea consumption, essential oil production, ornamental use, and seed oil production [7–9]. Seed oil from *C. japonica* L. is widely used in Asia for cooking, skincare, and hair health and has been reported to have beneficial biological health effects, including anti-inflammatory, improved lipid metabolism, and skin barrier function [10–12]. Seed cake is a by-product that remains after oil extraction and represents an underutilized resource with promising applications in the food and pharmaceutical industries, particularly in the upcycling of agricultural by-products [13]. Defatted *C. japonica* seed cake residue remaining after extracting seed oil is used for a variety of purposes, including fertilizer, soil conditioner, livestock feed, biological fuel, and raw material for organic cosmetics [14]. Although many studies have focused on the oil, flowers, and leaves of *C. japonica* and demonstrated their potential antibacterial, anti-oxidant, and anti-obesity activities [15–17], the potential of defatted seed cake remains unexplored. Recent studies have reported that defatted *C. japonica* seed cakes exhibit radical scavenging abilities, including NO and superoxide, and inhibit tyrosinase activity and melanin synthesis, indicating that antioxidant and skin-whitening effects depend on the extraction method (methanol vs. ethanol) [18]. Several studies have reported that defatted *C. japonica* seed cake contains various bioactive compounds such as saponins, amino acids, and flavonoid glycosides [19, 20]. In addition, defatted *C. japonica* seed cake has demonstrated several health benefits, including the protection of skin cells and hair growth [21, 22]. Additionally, we previously reported that the butanol (BuOH) fraction of defatted *C. japonica* L. seed cake exhibited the strongest in vitro radical scavenging activity among other extracts and fractions [23]. The health benefits of defatted *C. japonica* seed cakes have been the subject of numerous studies. However, no research has been conducted on the anti-inflammatory capacity of the BuOH fraction of defatted *C. japonica* L. seed cakes. In summary, seed cake remains underutilized despite its strong potential for use in food and pharmaceutical industries and its value as an upcycled agricultural resource. Therefore, identifying and characterizing the functional properties of this seed cake will provide a scientific basis for its development and application as a functional ingredient in food and pharmaceutical products.

In this study, lipopolysaccharide (LPS) was used to induce an inflammatory response in macrophages and mice to evaluate the anti-inflammatory effects of the BuOH fraction derived from defatted *C. japonica* L. seed cake. In addition, ultraperformance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC-QTOF MS/MS) was employed to identify and characterize the bioactive compounds present in this fraction.

2. Materials and Methods

2.1. Preparation of Samples. Defatted *C. japonica* L. seed cake was provided by the Korea Camellia Research Institute (Tongyeong, Korea). Defatted *C. japonica* L. seed cakes were subjected to extraction overnight in 80% ethanol. Following 3 h of reflux evaporation and heating at 70°C using an auxiliary rotary evaporator, the filtrate was collected and the procedure was repeated three times. To obtain the BuOH fraction of the defatted *C. japonica* L. seed cake with a yield of 0.29%, the filtrate was extracted five to six times using petroleum ether, ethyl acetate, and *n*-BuOH at various polarity gradients. The extract was then dried and evaporated using rotary evaporation at 80°C till crystallization.

2.2. Cell Culture. The mouse-derived macrophage cell line, RAW 264.7 (KCLB No. 40071), was obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured at 37°C with 5% CO_2 in a T75 flask with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin–streptomycin. When the cell confluence reached 80%, they were subcultured into three flasks.

2.3. Cell Viability. A 96-well plate was seeded with 100 μL culture media containing 5×10^5 cells at passage 3, which were allowed to grow for 24 h. After 24 h of cell culture, a concentration of 5, 10, 25, or 50 μg/mL was administered to 50 μL of each of the BuOH fractions from the defatted *C. japonica* L. seed cake. Four hours later, 5 μg/mL LPS was treated and incubated. The LPS concentration was set between 1 μg/mL and 10 μg/mL, based on the previous research findings that showed an increase in NO production within this range [24]. To facilitate comparison, two groups were established: one that had not been treated with LPS (labeled control, CON) and another that had been treated with LPS (labeled LPS). After 24 h, 0.5 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (#M6494, Invitrogen, Carlsbad, CA, USA) was added to each well, and the cells were incubated for 4 h. Purple insoluble formazan produced by live cells was solubilized in dimethyl sulfoxide, and its absorbance was analyzed at 560 nm using a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, Waltham, MA, USA). In comparison with the viability of cells in CON wells, viability of LPS-treated cells was calculated as %.

2.4. NO Production. Up to LPS treatment, the sample treatment process for assessing NO generation remained the same. Following 24-h LPS treatment, each well of the 96-well plate received 100 μL of the supernatant and 100 μL of the Griess reagent (#G4410, Sigma-Aldrich, St. Louis, MO, USA). After 15 min of incubation at room temperature (RT)

under light-shielded conditions, the absorbance at 540 nm was determined using a microplate reader.

2.5. Measurement of Inflammatory Cytokines. The levels of inflammatory cytokines in this study remained the same until LPS treatment of the cells. After 24-h LPS treatment, the supernatant was collected and analyzed using ELISA. The manufacturer's instructions were followed for all ELISA procedures. The levels of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α, #MTA00B), interleukin (IL)-6 (#M6000B-1), and IL-1β (#MLB00C) were measured using ELSA kit purchased from R&D Systems (Minneapolis, MN, USA).

2.6. Animal Experiments. Six-week-old male ICR mice (weighing 28–32 g) were purchased from Orient Bio (Seongnam, South Korea). All mice were maintained under controlled conditions of humidity (50 ± 10%), light (12-/12-h light/dark cycle), and temperature (20°C ± 2°C). The mice were randomized and transferred to plastic cages (four mice per cage) and provided ad libitum with water and a commercially available diet during the experimental period. Following a 7-day acclimation period, the mice were randomly divided into three groups ($n = 8$ per group): CON, LPS, and LPS + BuOH fraction of defatted *C. japonica* L. seed cake. They were then reared for 2 weeks. For 2 weeks, pure water was administered orally to the CON and LPS groups. In contrast, the LPS + BuOH fraction of defatted *C. japonica* L. seed cake group was orally administered with 50 mg/kg/day of the BuOH fraction from the defatted *C. japonica* L. seed cake (diluted in purified water) for the same duration. The administration concentration of LPS was determined based on the previous studies that modeled diet-related systemic inflammation [25]. After the 2-week intervention, the day before sacrifice, the CON mice were intraperitoneally injected with saline. The LPS and BuOH fractions from the defatted *C. japonica* L. + LPS groups were intraperitoneally administered LPS at a dose of 15 mg/kg/day. The mice were provided a regular meal (AIN-93G; Diets, Bethlehem, PA, USA) and unlimited access to freshwater during the entire experiment, with both being changed daily. The bedding and feed were removed 12 h prior to sacrifice, leaving only drinking water available. All the mice were euthanized via inhalation anesthesia using isoflurane. After anesthetizing the mice, their livers were excised, washed with 0.9% saline, and immediately submerged in liquid nitrogen. All samples were maintained at −80°C until further analysis. The Gyeongsang National University Institutional Animal Care and Use Committee evaluated and approved all the animal protocols (Approval No. GNU-231109-M0207).

2.7. Serum Biochemical Analysis. After completing the mouse experiment, blood was collected, and 100 μL of separated serum was loaded onto the reagent disk (General Chemistry I Lyophilized Kit, Tianjin MNCHIP Technologies Co., Ltd, China) and analyzed using the MNCHIP Pointcare M4 (Tianjin MNCHIP Technologies Co., Ltd) blood biochemistry analyzer. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were assessed using a blood biochemistry analyzer.

2.8. Hematoxylin and Eosin (H&E) Staining. H&E staining was performed to evaluate cell damage (neutrophil infiltration) [26]. The stained slides were examined under a microscope (EVOS M7000, Thermo Fisher Scientific) with four slides per group and 10 fields per slide, with no overlap. Liver tissues were fixed in 10% formalin for 72 h and embedded in paraffin blocks at RT in preparation for H&E staining. After the tissue was cut to a thickness of 5 μM using microtome, sections were mounted and attached to glass slides. The tissue sections were deparaffinized, rehydrated, and stained with hematoxylin for 5 min, followed by a 10-min rinse with water. Following decolorization with 1% HCl, the sections were rinsed with water for 10 min, stained with eosin for 1 min, and dehydrated with an alcohol gradient [27]. The sections were first cleared with xylene and then sealed with neutral gum.

2.9. Western Blotting. After completing the cellular experiments, RAW 264.7 cells and the growth medium were completely collected from the culture dish, placed into 50-mL conical tubes, and centrifuged at $38,772 \times g$ for 5 min at 4°C to obtain a pellet. The collected RAW 264.7 cells and the liver tissue from the mouse, which had been stored in a −80°C freezer, were used for western blotting. Cells and liver tissue were homogenized using a sonicator (Sonic and Material Vibra Cell VC600; Sonic and Material Inc., Danbury, CT, USA), using a bead beater (TissueLyser II; Qiagen, Tokyo, Japan). PRO-PREP protein extraction solution (Intron Biotechnology, Seongnam, Korea) was used for homogenization. The homogenized samples were centrifuged, and the supernatant was collected and quantified using the Bradford assay. Western blotting was performed as previously described [28, 29]. Proteins isolated from cells were analyzed, including pNF-κB (#sc-136548, Santa Cruz Biotechnology, Dallas, TX, USA), iNOS (#13120, Cell Signaling, Danvers, MA, USA), and COX-2 (#12282, Cell Signaling), and normalized to β-actin (#8457, Cell Signaling). In proteins isolated from the liver, IκBα (#sc-1643, Santa Cruz Biotechnology), NF-κB (#6965, Cell Signaling), iNOS (#13120, Cell Signaling), COX-2 (#12282, Cell Signaling), IL-1β (#sc-52012, Santa Cruz Biotechnology), and IL-6 (#sc-32296, Santa Cruz Biotechnology) were analyzed and normalized to GAPDH (#2118, Cell Signaling).

2.10. Quantitative Real-Time RT-PCR. AccuPower RT Premix (Bioneer, Daejeon, Korea) was used to synthesize cDNA from the total liver RNA extracted using TRIzol reagent (Ambion, Carlsbad, CA, USA) by following the manufacturer's instructions. In a 96-well plate (MicroAmp 96-Well Reaction Plate with Barcode, Applied Biosystems, Foster City, CA, USA) that contained Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), the

cDNA was mixed with forward and reverse primers for qPCR. The ViiA7 real-time PCR system (Applied Biosystems) was used for further analysis. The relative quantification $2^{-\Delta\Delta C(t)}$ method was used to assess the cycle threshold (Ct) values that were measured following the experiment. The primer sequences used in the study are listed in Table 1.

2.11. UPLC-QTOF-MS Analysis. A Nexera XS UPLC system (Shimadzu; Kyoto, Japan) was used to analyze the compounds in the defatted cake of Camellia seeds. The detector was coupled with an X500R QTOF mass spectrometer (SCIEX, Framingham, MA, USA). For chromatographic separation, a Zorbax Eclipse plus C18 column ($2.1 \times 100\,\text{mm} \times 1.8\,\mu\text{m}$) maintained at 40°C was used. The mobile phase composition was 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The injection volume was 10 μL and elute time 52 min with a flow rate of 0.3 mL/min. The gradient elution was designed as follows: 0–2 min, 1%–10% (B); 4–10 min, 10%–20% (B); 10–12 min, 20%–30% (B); 12–20 min, 30%–30% (B); 20–22 min, 30%–50% (B); 22–30 min, 50%–50% (B); 32–40 min, 50%–70% (B); 40–42 min, 70%–90% (B); and 42–52 min, 90% (B). The mass spectrometric analyses were conducted at the positive ion mode (ESI+) with an ion spray voltage of 5500 V and a capillary temperature of 500°C. The pressure of the curtain gas was 30 psi, and the pressure of Ion Source Gas 1 and Ion Source Gas 2 was 50 psi. In addition, the QTOF MS range used was 100–1500 m/z, and the QTOF MS/MS range used was 50–1500 m/z at a collision energy of 45 ± 15 V. SCIEX OS software (Version 3.0.0.3339) was used to gather the raw data files from UPLC-QTOF/MS analysis. Finally, the peak selection retention time was set to 0–20 min.

2.12. Statistical Analysis. One-way ANOVA and Tukey's post hoc tests were used to statistically examine the research outcomes. GraphPad Prism Version 8 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of BuOH Fraction From C. japonica L. Seed Cake on NO Production in LPS-Treated Macrophages. To evaluate the cytotoxicity of the BuOH fraction from defatted *C. japonica* L. seed cake on RAW 264.7 cells, cell viability was investigated. Cells were exposed to 5–50 μg/mL of BuOH fraction from defatted *C. japonica* L. seed cake. As illustrated in Figure 1(a), cell viability in the treatment of BuOH fraction from defatted *C. japonica* L. seed cake at concentrations ranging from 5 to 50 μg/mL did not differ from the control group. This finding showed that the BuOH fraction from defatted *C. japonica* L. seed cake did not cause cytotoxicity at doses ranging from 5 to 50 μg/mL. As shown in Figure 1(b), the LPS group produced more NO than the CON group ($p < 0.0001$). However, NO production was significantly lower in all groups treated with BuOH fraction from defatted *C. japonica* L. seed cake compared to the LPS group (5 μg/mL, $p < 0.01$; 10 μg/mL, $p < 0.001$; both 25 μg/mL and 50 μg/mL, $p < 0.0001$). The NO production levels in each BuOH fraction from defatted *C. japonica* L. seed cake-treated group were as follows: CON, 100 ± 1.72%; LPS, 152.26 ± 5.43; 5 μg/mL, 142.31 ± 6.06; 10 μg/mL, 140.70 ± 0.93; 25 μg/mL, 129.25 ± 3.14%; and 50 μg/mL, 120.52 ± 2.74%. Treatment with the BuOH fraction of defatted *C. japonica* L. seed cake was found to have a protective effect against the LPS-induced inflammatory response.

3.2. Effects of BuOH Fraction From Defatted C. japonica L. Seed Cake on the Inflammatory Cytokines in Macrophages Induced by LPS. The ELISA findings demonstrated that treating LPS considerably raised the secretion of the inflammatory cytokines TNF-α, IL-6, and IL-1β (CON vs. LPS, $p < 0.0001$). The released TNF-α was significantly lower in all groups treated with BuOH fraction from defatted *C. japonica* L. seed cake compared to the group with LPS alone ($p < 0.0001$) (Figure 2(a)). In comparison with the LPS group, the groups of BuOH fraction from defatted *C. japonica* L. seed cake with concentrations of 25 μg/mL ($p < 0.05$) and 50 μg/mL ($p < 0.0001$) showed a notably decreased release of IL-6 (Figure 2(b)). The secretion tendency of IL-1β was similar to that of IL-6 (Figure 2(c)).

3.3. Effects of BuOH Fraction From Defatted C. japonica L. Seed Cake on the Inflammatory Cell Signaling in LPS-Induced Cells. Western blotting was used to determine whether the BuOH fraction from the defatted *C. japonica* L. seed cake inhibited the protein levels of pNF-κB, iNOS, and COX-2 in LPS-treated cells. As illustrated in Figure 3, the pNF-κB, iNOS, and COX-2 protein levels were all significantly higher with treatment of LPS than those in the CON group ($p < 0.0001$). In contrast, with the treatment of the BuOH fraction from defatted *C. japonica* L. seed cake at 10–50 μg/mL, the protein levels of pNF-κB and COX-2 showed a dose-dependent decrease, in comparison with the LPS group. Additionally, iNOS protein expression was significantly decreased at 25 μg/mL ($p < 0.01$) and 50 μg/mL ($p < 0.0001$) concentrations of the BuOH fraction.

3.4. Effects of BuOH Fraction From Defatted C. japonica L. Seed Cake on Liver Damage in LPS-Treated Mice. The animal experimental schedule is shown in Figure 4(a). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were higher in the LPS group than in the CON group ($p < 0.05$). However, no notable differences were observed between the other groups for the BuOH fraction from the defatted *C. japonica* L. seed cake-administered group (Figures 4(b) and 4(c)). Neutrophil infiltration, which indicates liver damage, was confirmed only in the liver of the LPS group, but not in the CON or the BuOH fraction from defatted *C. japonica* L. seed cake + LPS group (Figure 4(d)). Statistical analysis could not be performed for the control or BuOH fraction from the defatted

Table 1: Primer sequences of target genes for quantitative real-time reverse transcription polymerase chain reaction analysis.

Gene symbol	Forward primer (5′ ⟶ 3′)	Reverse primer (5′ ⟶ 3′)
Gapdh	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
Il1b	GCAACTCTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Tnf	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT

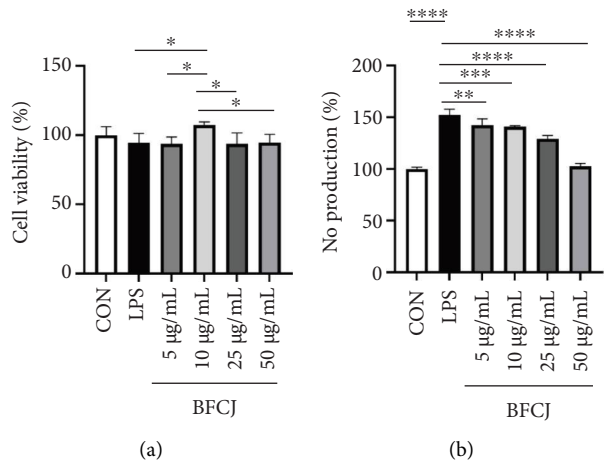


Figure 1: Effects of BuOH fraction from defatted *C. japonica* L. seed cake (BFCJ) on cell viability (a) and NO production (b) in LPS-treated RAW 264.7 cells. All data are presented as the mean (M) ± standard deviation (S.D.). The M ± S.D was calculated using six triplicate wells for each group ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

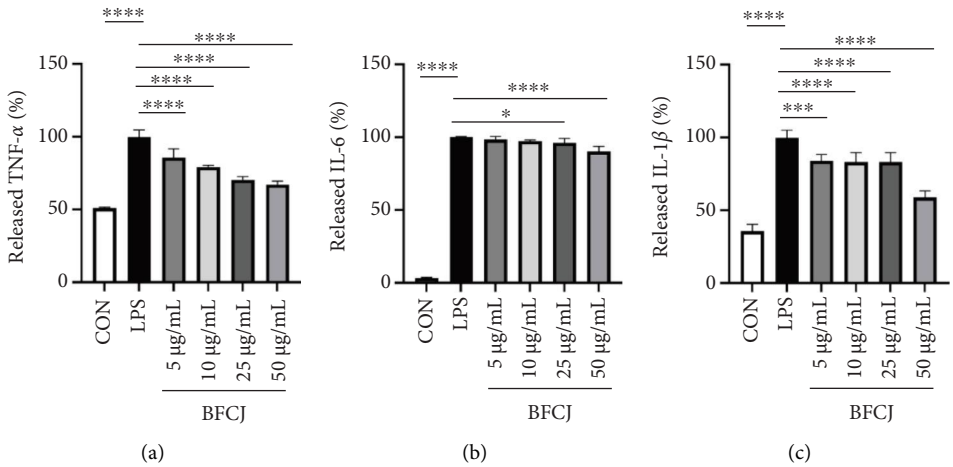


Figure 2: Effects of BuOH fraction from defatted *C. japonica* L. seed cake (BFCJ) on the production of inflammatory cytokines such as TNF-α (a), IL-6 (b), and IL-1β (c) in LPS-treated RAW 264.7 cells. All data are presented as the M ± S.D ($n = 6$). The M ± S.D was calculated using six triplicate wells for each group. $^{*}p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

C. japonica L. seed cake-administered group because the mean and standard deviation were both zero. In the LPS group, an average of 1.32 neutrophil infiltrations were confirmed per field.

3.5. Effects of BuOH Fraction From Defatted C. japonica L. Seed Cake on Inflammatory Cytokines and Signaling in the Liver of Mice Treated With LPS. The levels of pro-inflammatory cell signaling proteins in the mouse liver were analyzed using western blotting. The LPS-treated group exhibited notably increased expression of all pro-inflammatory cell signaling proteins (pNF-κB, $p < 0.0001$; COX-2, $p < 0.0001$; iNOS, $p < 0.05$; IL-1β, $p < 0.001$; and IL-6, $p < 0.0001$), compared with the CON. Conversely, the expression of these pro-inflammatory cell signaling proteins in the BuOH fraction from defatted *C. japonica* L. seed cake-administered group was significantly lower than that in the LPS-treated group (pNF-κB, $p < 0.0001$; COX-2, $p < 0.0001$; iNOS, $p < 0.0001$; IL-1β, $p < 0.001$; IL-6, $p < 0.0001$) (Figure 5).

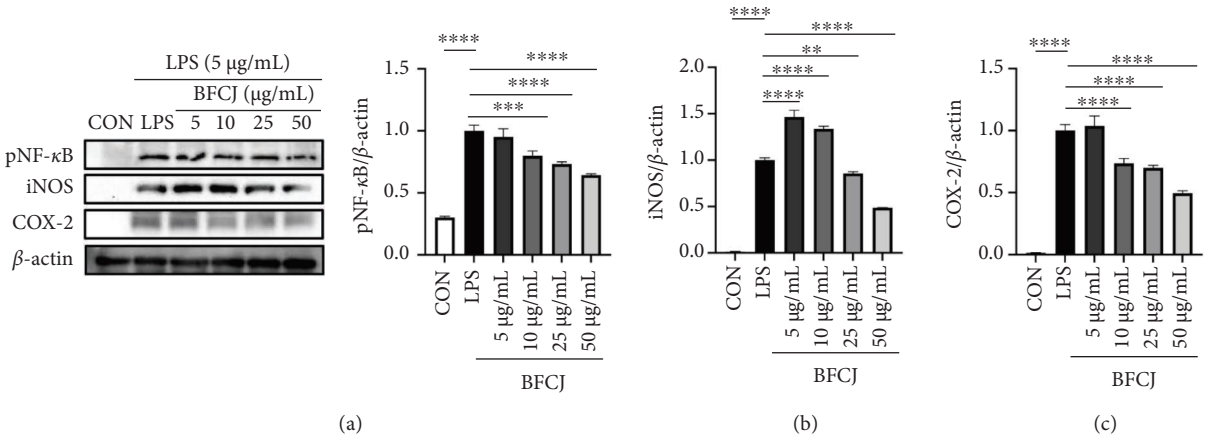


Figure 3: Effects of BuOH fraction from defatted *C. japonica* L. seed cake (BFCJ) on the protein expressions of pNF-κB (a), iNOS (b), and COX-2 (c) in LPS-treated cells. All data are presented as the M ± S.D ($n = 6$). The M ± S.D was calculated by using six triplicate wells for each group. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

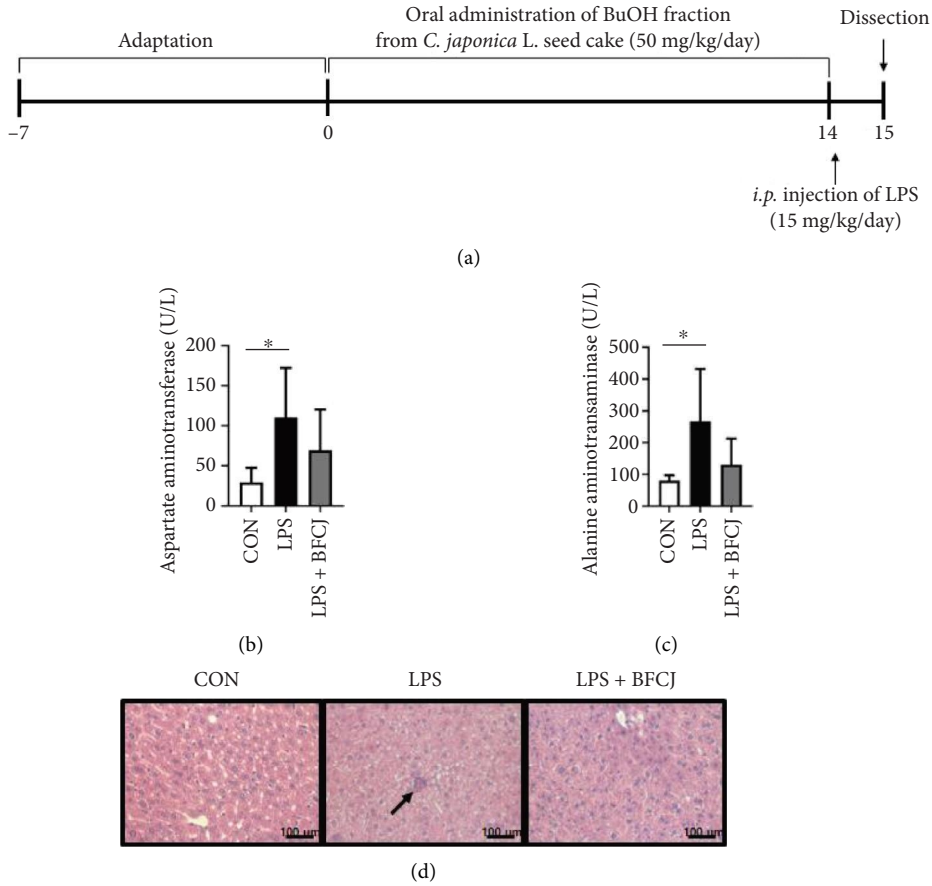


Figure 4: Experimental schedule (a). Effects of BuOH fraction from defatted *C. japonica* L. seed cake (BFCJ) on the serum AST (b), ALT (c), and histological changes of the liver (d) in LPS-induced mice. All data are presented as the M ± S.D ($n = 6$). The M ± S.D was calculated by capturing 10 nonoverlapping random fields per slide from four slides in each group using microscope. $^{*}p < 0.05$. The arrow indicates neutrophil infiltration.

The expression of pro-inflammatory cell signaling genes in the liver showed the same trend as the protein expression results. The *Il1b* gene, which encodes for IL-1β, was notably more expressed in the LPS group, compared to the CON group ($p < 0.05$), and significantly lower in the BuOH fraction from defatted *C. japonica* L. seed cake-administered

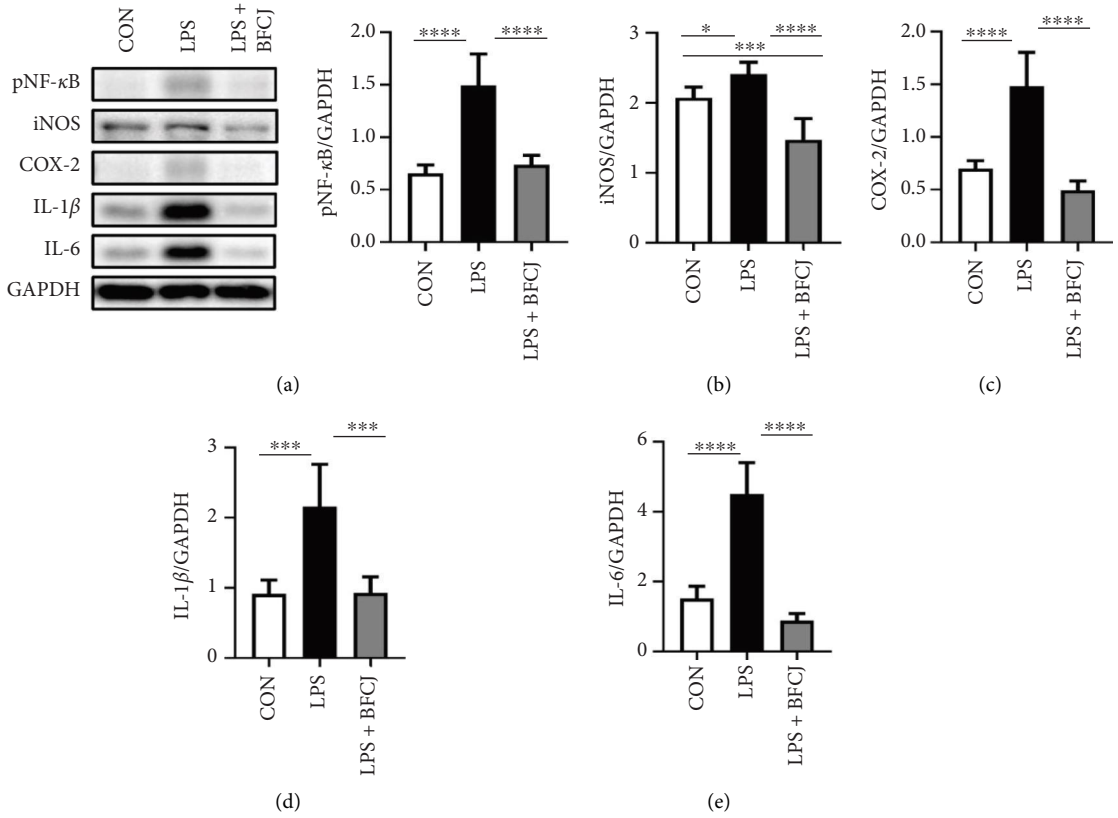


Figure 5: Effects of BuOH fraction from defatted *C. japonica* L. seed cake (BFCJ) on the protein expression of pNF-κB (a), iNOS (b), COX-2 (c), IL-1β (d), and IL-6 (e) in the liver of LPS-induced mice. All data are presented as the M ± S.D ($n = 6$). The M ± S.D was calculated using proteins extracted from six mouse liver samples per group for western blot analysis. $^{*}p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

group compared to the LPS group ($p < 0.05$). The LPS-treated ($p < 0.0001$) and the BuOH fraction from the defatted *C. japonica* L. seed cake-administered ($p < 0.05$) groups showed notably higher expression of the *Il6* gene, which encodes IL-6, than the CON group. However, the BuOH fraction from the defatted *C. japonica* L. seed cake-administered group showed notably lower gene expression than the LPS group ($p < 0.0001$). The LPS group showed relatively higher *Tnf* (encoding TNF-α) expression than the CON group ($p < 0.05$), while the LPS+BFCJ group exhibited relatively lower expression compared with the LPS group ($p < 0.05$) (Figure 6).

3.6. Chemical Characterization of BuOH Fraction From Defatted C. japonica L. Seed Cake Using UPLC-QTOF-MS/MS. As shown in Figure 7, we analyzed the base peak chromatogram of the BuOH fraction from defatted *C. japonica* L. seed cake in the positive ion mode using UPLC-QTOF-MS/MS for up to 20 min. The compounds identified in these collections are summarized in Table 2. Eight chromatographic peaks were detected and tentatively identified. Their retention times and MS characteristics, including accurate mass, isotopic distribution, and fragmentation patterns in the positive ionization mode, were compared with the previously reported data. Peak 1 was identified as dihydrokaempferol, based on its fragmentation pattern at m/z 289.0722 in the $[M + H]^+$ ion (PubChem CID 122850). Peak 2 showed the $[M + H]^+$ ion at m/z 859.2497, which seemed to be a kaempferol tetraglycoside that probably contains a kaempferol, a glucose, a rhamnose, and two xyloses. Peak 3 had a $[M + H]^+$ ion at m/z 889.2569, which also seemed to be a kaempferol tetraglycoside that may contain a kaempferol, two glucoses, a rhamnose, and a xylose. Peaks 4 and 5 showed the $[M + H]^+$ ion with an m/z value of 727.2048 and 757.2056, respectively, which were tentatively identified as kaempferol 3-[2G-xylosylrutinoside] and kaempferol 3-[2G-glucosylrutinoside] [30]. Peak 6 showed an $[M + H]^+$ ion with an m/z value of 433.1146, which was tentatively identified as apigenin-6-C-glucoside (PubChem CID 162350). Peak 7 showed an $[M + H]^+$ ion with an m/z value of 741.2264 and was tentatively identified as kaempferol-3-O-robinoside-7-O-rhamnoside (PubChem, CID 5281693). Peak 8 had an $[M + H]^+$ ion with an m/z value of 595.1645 and was tentatively identified as kaempferol-3-O-rutinoside (PubChem CID 5318767).

4. Discussion

Research is ongoing to explore diverse strategies to enhance the utility of defatted *C. japonica* L. seed cakes. Typically, *C. japonica* L. seeds are processed to extract oil, after which

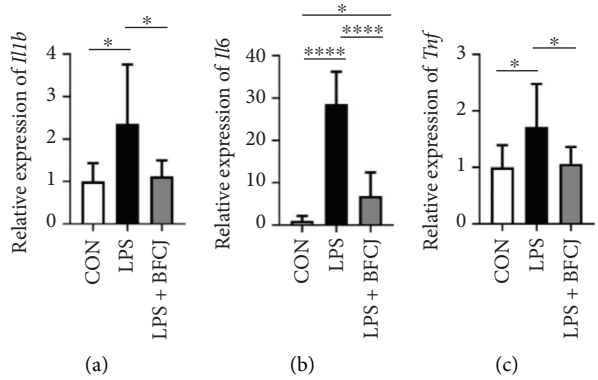


Figure 6: Effects of BuOH fraction from defatted *C. japonica* L. seed cake (BFCJ) on the gene expression of *Il1b* (a), *Il6* (b), and *Tnf* (c) in the liver of mice induced by LPS. All data are presented as the M ± S.D ($n = 6$). RNA extracted from six mouse liver samples per group was used for cDNA synthesis, followed by quantitative real-time reverse transcription polymerase chain reaction, after which the M ± S.D was calculated. $^{*}p < 0.05$ and $^{****}p < 0.0001$.

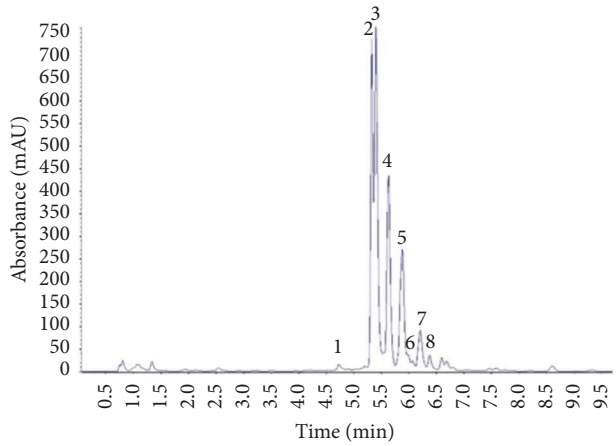


Figure 7: UPLC-QTOF-MS/MS base peak chromatogram of BuOH fraction from defatted *C. japonica* L. seed cake (BFCJ). Peak numbering follows that stated in Table 1.

the resulting seed cake constitutes approximately 40%–50% of the initial weight [31]. This residual defatted seed cake, rich in protein and fiber, has potential as animal feed, particularly for ruminants such as cows and sheep [32, 33]. Additionally, owing to its high organic content, it can serve as a soil conditioner, enhancing soil fertility, increasing water retention capacity, and stimulating the activity of beneficial microorganisms [34]. Despite the various applications of defatted *C. japonica* L. seed cakes, improper handling can lead to environmental issues. For instance, greenhouse gases such as methane may be emitted during decomposition, and leachate from the decomposition process can contaminate groundwater or adjacent aquatic systems [35]. Moreover, the accumulation of substantial quantities of waste requires extensive space for storage or processing, potentially leading to financial losses. Furthermore, failure to effectively utilize these by-products may result in the squandering of resources. Thus, appropriate recycling or processing of defatted *C. japonica* L. seed cake is a crucial endeavor that supports both environmental sustainability and economic viability.

To maximize the value derived from *C. japonica* L. seed cake, we explored the extraction of active compounds using BuOH as the solvent. BuOH is a less polar solvent than ethanol, and the BuOH extract has the advantage of being able to selectively extract components that have a polarity far from that of water [36]. This indicates that the BuOH extracts are more likely to contain fat-soluble components than the ethanol extracts, which may also have anti-inflammatory and health-promoting activities. Therefore, the BuOH fraction from defatted *C. japonica* L. seed cakes requires separate verification of its efficacy. If the efficacy of the defatted *C. japonica* L. seed cake is verified, it would be desirable to use both ethanol and BuOH as solvents in the future when extracting active ingredients from defatted *C. japonica* L. seed cake. In this study, the phenolic compounds from the BuOH fraction were separated and identified using UPLC-QTOF-MS/MS [37]. Although the substances contained in the BuOH fraction from defatted *C. japonica* L. seed cake were not definitively confirmed through the comparison with the reference standards in our results, UPLC-QTOF-MS analysis of the BuOH fraction from defatted *C. japonica* L. seed cake allowed the identification of eight suspected flavonoids. In our study, dihydrokaempferol (Peak 1), kaempferol 3-[2G-xylosylrutinoside] (Peak 4), kaempferol 3-[2G-glucosylrutinoside] (Peak 5), apigenin-6-C-glucoside (Peak 6), kaempferol-3-O-robinobioside-7-O-rhamnoside (Peak 7), and kaempferol-3-O-rutinoside (Peak 8), which are presumed to be the main compounds in BuOH fraction from defatted *C. japonica* L. seed cake, have been shown to been reported to exert anti-inflammatory effects in various studies. Dihydrokaempferol reduces inflammation by blocking pro-inflammatory cytokines such as IL-6 and TNF-α [38, 39]. Apigenin-6-C-glucoside, also known as isovitexin, is a derivative of apigenin with significant anti-inflammatory activity. Isovitexin reduces inflammation by inhibiting the production of inflammatory cytokines, iNOS and COX-2, to exert anti-inflammatory effects that are comparable to those of its isomer, vitexin. These actions help reduce inflammation, particularly in conditions such as

Table 2: Compounds identified in the BuOH fraction from defatted *C. japonica* L. seed cake by UPLC-QTOF-MS/MS.

Peak no	Retention time (min)	Compounds	Adduct	Observed mass (m/z)	Major fragments
1	4.84	Dihydrokaempferol	$[M+H]^+$	289.0722	153, 271, 288, 289
2	5.35	Kaempferol + glucose + rhamnose	$[M+H]^+$	859.2497	85, 145, 163, 287, 449
3	5.45	Kaempferol + 2 glucose + rhamnose + xylose	$[M+H]^+$	889.2569	85, 145, 163, 279, 287, 449
4	5.67	Kaempferol 3-[2G-xylosylrutinoside]	$[M+H]^+$	727.2048	85, 129, 147, 287, 449
5	5.93	Kaempferol 3-[2G-glucosylrutinoside]	$[M+H]^+$	757.2056	73, 149, 287, 449
6	6.01	Apigenin-6-C-glucoside	$[M+H]^+$	433.1146	313, 415, 433, 434, 435
7	6.27	Kaempferol 3-O-robinoside-7-O-rhamnoside	$[M+H]^+$	741.2264	287, 741, 742
8	6.42	Kaempferol-3-O-rutinoside	$[M+H]^+$	595.1645	287, 595

neuroinflammation and cardiovascular disease. The anti-inflammatory properties of isovitexin are likely mediated through pathways such as NF-κB, MAPK, and STAT3, which are involved in inflammatory processes. By targeting these pathways, isovitexin can reduce inflammation in various tissues and may be beneficial for conditions such as arthritis, atherosclerosis, and certain types of cancers [40, 41]. Kaempferol-3-O-rutinoside possesses strong anti-inflammatory, anti-oxidant, and antitumor activities. It specifically lowers inflammation by suppressing inflammatory enzymes such as COX-2, which was also confirmed in our study. Although kaempferol-3-O-robinobioside-7-O-rhamnoside has been studied less extensively, its structural similarity to other kaempferol glycosides suggests potential anti-inflammatory effects. Moreover, Peaks 4 and 5 exhibited different $[M+H]^+$ ions at m/z 727.2048 and m/z 757.2056, respectively, and showed the same major fragments at m/z 287 and m/z 449, respectively. The fragment ion at m/z 449 was produced by a loss of rhamnose and xylose, and m/z 287 corresponded to $[\text{kaempferol}+H]^+$. Besides, Peak 4 showed fragmentation ions at m/z 129 and m/z 147, which may be due to the loss of hydroxyl groups [30]. The fragment ion at m/z 449 has been reported to represent kaempferol glucoside; therefore, Peaks 4 and 5 were tentatively identified as flavonoid glucosides—kaempferol 3-[2G-xylosylrutinoside] and kaempferol 3-[2G-glucosylrutinoside], respectively [30]. In addition, Peaks 2 and 3 with the fragment ions at m/z 859.2497 and m/z 889.2569, respectively, have never been revealed in previous studies; yet they were likely to be kaempferol tetraglycosides. Although there are few studies on kaempferol 3-[2G-xylosylrutinoside] and kaempferol 3-[2G-glucosylrutinoside], or the kaempferol tetraglycosides, it has been reported that flavonoids with disaccharide and monosaccharide, or trisaccharide exhibit varying degrees of anti-inflammatory activity [42]. The detection of these kaempferol glycosides highlights their role as major components of the BuOH fraction of *C. japonica* seed cake. These glycosides are typically more polar and water-soluble, which may explain their preferential partitioning into the BuOH fraction during solvent extraction. These compounds also more likely contribute significantly to the observed anti-inflammatory effects. Given the identification of these major components of the BuOH fraction, further studies are needed to better understand their therapeutic potential and specific biological roles. Kaempferol glycosides generally exhibit these effects by inhibiting inflammatory factors such as NO and cytokines.

We discovered that the BuOH fraction from defatted *C. japonica* L. seed cake had the potential to increase cell viability and enhance anti-inflammatory capacity (Figure 1). This evidence also indicated that it possesses anti-inflammatory properties (Figure 2). Specifically, the anti-inflammatory effect seems to occur by inhibiting cell signaling pathways related to inflammation, namely, pNF-kB, iNOS, and COX-2 (Figure 3). When there is an inflammatory stimulus in cells, NF-κB is activated and transfers to the nucleus, where it stimulates the transcription of numerous genes linked to inflammation, such as iNOS and COX-2 [43]. Western blot analysis confirmed an increase in the protein expression upon stimulation of this signaling pathway. We confirmed that treatment with the BuOH fraction from defatted *C. japonica* L. seed cake suppressed the LPS-induced inflammatory response due to LPS (Figure 3). Although the direct effects of p65 and p50 were not evaluated in this study, the evaluation of inflammatory mediators such as IL-6, iNOS, COX-2, and IL-1β allowed for an indirect assessment of NF-κB pathway activation. These molecules are key inflammatory mediators regulated by the NF-κB pathway, and their expression levels reliably affect pathway activation. Therefore, the analysis of these cytokines and enzymes served as an alternative approach for evaluating NF-κB pathway activation. However, according to our results, the BuOH fraction from defatted *C. japonica* L. seed cakes may exhibit significant anti-inflammatory activity at high concentrations.

We conducted animal experiments to assess whether the BuOH fraction from defatted *C. japonica* L. seed cake ingestion could replicate the anti-inflammatory effects observed in cell-based studies. Although no significant differences were found, the BuOH fraction from defatted *C. japonica* L. seed cake demonstrated a tendency to protect against LPS-induced liver damage, as indicated by blood markers and histological analysis (Figure 4). Although we initially considered implementing histopathological scoring to quantify liver damage, neither the CON nor the LPS + BFCJ group exhibited observable lesions or inflammatory infiltration. Consequently, their scores yielded means and standard deviations of zero, rendering the statistical analyses unreliable. To maintain scientific rigor and avoid misinterpretation, we decided not to include the histological scoring of the liver. In addition, similar to the

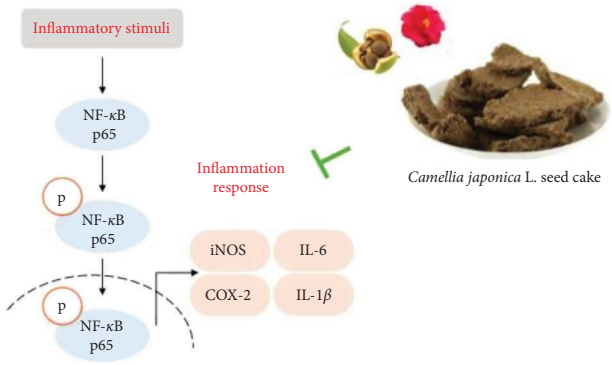


FIGURE 8: Summary of the anti-inflammatory effects of the BuOH fraction from defatted *C. japonica* L. seed cake.

cellular experiments, both the protein and gene expression modulated the inflammatory pathway (Figures 5 and 6).

In this study, a high dose of LPS (15 mg/kg, *i.p.*) was used to establish mouse models of acute inflammation. Such high-dose LPS injection is a well-known trigger of moderate to severe systemic inflammation to induce endotoxemia, resulting in tissue dysfunction and damage. Previous studies have demonstrated that high-dose LPS (10, 15, or 20 mg/kg) effectively increases the levels of serum TNF-α and IL-6, and macrophages and neutrophils within 24 h [44–46]. Moreover, high-dosing LPS exposure tends to induce endotoxemia and increase with time to release inflammatory cytokines [47]. However, future studies should consider using lower or repeated LPS doses, which may more accurately reflect the pathophysiology of metabolic endotoxemia and more relevant for evaluating the anti-inflammatory effects of functional foods [25].

Previous studies have reported a wide range of in vivo doses for different parts of *C. japonica*, demonstrating therapeutic effects without observable toxicity. For instance, oral administration of the *C. japonica* leaf extract at 300 mg/kg showed antinociceptive effects by regulating maladaptive changes in a chronic constriction injury pain-induced Sprague-Dawley rats, while doses of 30–300 mg/kg alleviated potassium oxonate-induced hyperuricemia in mice [48, 49]. In a rat model of the cerebral ischemia model, the flower extract of *C. japonica* at 20, 40, and 80 mg/kg significantly inhibited learning and memory deficits [50]. In addition, seed cake ethanol extracts of *C. japonica* administered at 250 and 1000 mg/kg in a high-fat diet-induced mouse model of metabolic dysfunction-associated steatotic liver disease enhanced mitochondrial function and alleviated metabolic dysfunction, suggesting potential benefits for liver disease [51]. According to these reports, a wide range (20–1000 mg/kg) of *C. japonica* parts, including the leaves, flowers, and seed cake, has been used in animal models. Given that the present study is the first to investigate the BuOH fraction from defatted *C. japonica* seed cake, which is enriched in bioactive constituents compared with crude extracts, we selected 50 mg/kg/day as a safe and moderate dose to evaluate its anti-inflammatory effects in an LPS-induced mouse model. In preclinical studies, establishing drug exposure in animal models is essential for assessing safety evaluation and provides a basis for predicting dose levels in toxicology studies [52]. Dose selection in animal models can range from subtherapeutic to overtly toxic levels, and single-dose evaluations are often used as preliminary dose-range finders to help identify the maximum tolerated dose [52]. Additionally, single-dose studies in relevant species can provide insights into potential effects on target organs [53]. However, single-dose studies have inherent limitations, particularly in their inability to fully characterize dose–response relationships and safety profiles. This limitation should be considered when interpreting the current findings. Regarding to this limitation, future work is including studies using multiple dose levels of BFCJ to identify the dose–response relationship and establish a more comprehensive safety profile.

Numerous bioactive effects of *C. japonica* L. have been confirmed in several studies, highlighting the potential of using by-products remaining after food processing in beneficial ways. This supports effective recycling and disposal strategies for improving environmental sustainability and economic viability. However, there have been no studies, such as ours, that confirmed the activity of the remaining seed cake after using *C. japonica* L. and proved it through animal testing. Our study is the first to confirm that orally administered camellia seed cake extract is nontoxic and has anti-inflammatory effects.

5. Conclusions

In conclusion, the BuOH fraction from defatted *C. japonica* L. seed cake significantly attenuated NO production and inflammatory cytokine expression by modulating NF-κB signaling in LPS-induced RAW 264.7 macrophages. Furthermore, oral administration of this fraction ameliorated LPS-induced liver damage in mice by downregulating inflammatory cytokines and NF-κB signaling. These findings suggest that the BuOH fraction from defatted *C. japonica* seed cake is a potential anti-inflammatory agent (Figure 8).

Data Availability Statement

The data that support the findings of this study are available in the supporting information of this article.

Disclosure

All authors read and approved the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Kyung-Wan Baek: writing–original draft, visualization, validation, and formal analysis. Mei Tong He: writing–review and editing, visualization, validation, and data curation. Weon Pho Park: investigation and resources. Yu Ri Jeong: formal analysis. Ying-Ying Xiang: formal analysis. Jong-Hwa Won: formal analysis. Jine Shang Choi:

writing–review and editing, supervision, and conceptualization. Ji Hyun Kim: writing–review and editing, data curation, supervision, and conceptualization. Kyung-Wan Baek and Mei Tong He are the co-first authors. Kyung-Wan Baek and Mei Tong He contributed equally to this study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. *(Supporting Information)*

Figure S1: MS/MS spectra for Peak 2.
Figure S2: MS/MS spectra for Peak 3.
Figure S3: MS/MS spectra for Peak 4.
Figure S4: MS/MS spectra for Peak 5.

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