

Broadleaf *Mahonia attenuates granulomatous lobular mastitis-associated inflammation by inhibiting CCL-5 expression in macrophages*

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Abstract. Granulomatous lobular mastitis (GLM) is a type of chronic mammary inflammation with unclear etiology. Currently systematic corticosteroids and methotrexate are considered as the main drugs for GLM treatment, but a high toxicity and risk of recurrence greatly limit their application. It is therefore an urgent requirement that safe and efficient natural drugs are found to improve the GLM prognosis. Broadleaf *Mahonia* (BM) is a traditional Chinese herb that is believed to have anti-inflammatory properties according to ancient records of traditional Chinese medicine. The present study investigated this belief and demonstrated that BM significantly inhibited the expression of interleukin-1 β (IL-1 β), IL-6, cyclooxygenase-2 and inducible nitric oxide synthase in

RAW264.7 cells, but had little influence on the cell viability, cell cycle and apoptosis. Meanwhile, the lipopolysaccharide-induced elevation of reactive oxygen species and nitric oxide was also blocked following BM treatment, accompanied with decreased activity of nuclear factor- κ B and MAPK signaling. A cytokine array further validated that BM exhibited significant inhibitory effects on several chemoattractants, including chemokine (C-C motif) ligand (CCL)-2, CCL-3, CCL-5 and secreted tumor necrosis factor receptor 1, among which CCL-5 exhibited the highest inhibition ratio in cell and clinical GLM specimens. Collectively, the results show that BM is a novel effective anti-inflammatory herb *in vitro* and *ex vivo*, and that CCL-5 may be closely associated with GLM pathogenesis.

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Abbreviations: GLM, granulomatous lobular mastitis; BM, broadleaf *Mahonia*; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; ROS, reactive oxygen species; NO, nitric oxide; sTNFR1, secreted tumor necrosis factor receptor 1; iNOS, inducible nitric oxide synthase; NF- κ B, nuclear factor- κ B; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; TCM, traditional Chinese medicine

Key words: granulomatous lobular mastitis, macrophages, chemokine (C-C motif) ligand-5, inflammation, broadleaf *Mahonia*, nuclear factor- κ B/ mitogen-activated protein kinases

Introduction

Granulomatous lobular mastitis (GLM), first defined by Kessler and Woollock in 1972, is a rare, benign and chronic inflammatory lesion of the breast (1). The condition is characterized by painful breast masses, abscesses, draining sinuses and scarring (2). Dermatitis contusiformis is also reported to be a common clinical symptom in the majority of cases (3). Various factors have been reported to be associated with the occurrence of GLM, including breast feeding, microbiological infection, autoimmune diseases, smoking and a high level of prolactin (4). The natural course of GLM is relatively long. If no treatment is administered, the mean duration of spontaneous healing is 14.5 months in 50% of patients, and common sequelae include scarring, retraction of the skin and nipple, and shrinkage of the whole breast. Meanwhile, ~50% of patients experience recurrence after systematic therapy (1).

Clinically, it is difficult to differentiate GLM from other mammary diseases, including breast cancer, subareolar abscesses, plasma cell mastitis, mammary tuberculosis, fat

necrosis and sarcoidosis (5). Histological examination is the only golden criteria able to make a diagnosis. In the perspective of histology, GLM is characterized by lobular granuloma consisting of macrophages, neutrophils, lymphocytes, monocytes and lesions of non-cheese-like necrosis (6). Since granuloma is induced by macrophage infiltration and proliferation, macrophage activation is considered to be the central pathogenesis of GLM. Therapeutically, no standard regimen or guideline exists for GLM, but systematic corticosteroids and methotrexate have been used as primary treatments subsequent to surgery to prevent recurrence (6). Antibiotics or anti-tuberculotics have also been recommended for GLM therapy in certain patients (7). However, side effects, including antibiotic resistance, immunosuppression, hormone-induced concentric obesity and metabolic disorders, greatly limit the chronic application of current strategies. Meanwhile, breast reconstruction is usually necessary after surgery in severe GLM cases. Therefore, it is a necessary and urgent requirement to find natural anti-inflammatory agents with low toxicity for GLM therapy.

Macrophages serve important roles in the innate and adaptive immune responses by releasing various factors, including interleukin-6 (IL-6), IL-1 β , cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). All these mediators contribute to the inflammatory disease pathogenesis and are recognized biomarkers for evaluating the severity of inflammation. Nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways are considered as the upstream signaling controlling the transcription of the aforementioned inflammatory factors (8,9). p50/p65 heterodimer is the most highly characterized NF- κ B complex, which is kept inactive in the cytoplasm of resting cells. At NF- κ B activation, its translocation to the nucleus results in the promotion of downstream gene transcription. MAPK family members, including p38 MAPK, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), become activated following lipopolysaccharide (LPS) stimulation, and MAPK suppression results in the blockage of the secretion of various inflammatory cytokines. Meanwhile, MAPKs and NF- κ B can collaborate synergistically to induce the expression and secretion of pro-inflammation cytokines (10). Therefore, therapies targeting NF- κ B and MAPKs have become an important strategy for curing inflammatory diseases.

Traditional Chinese medicine (TCM) has a unique focus on in treating chronic inflammatory wounds and diseases. A number of studies have demonstrated that numerous Chinese herbs, including *Tripterygium wilfordii*, honeysuckle and dandelion, could exert anti-inflammatory effects via blocking reactive oxygen species (ROS) burst, balancing dysregulated immune cells or cytokines, inhibiting NF- κ B signaling and interrupting nitric oxide (NO) synthesis (11,12). Broadleaf *Mahonia* (BM) is a traditional Chinese herb that is widely distributed in the majority of regions worldwide. Although it is recorded that BM is capable of fighting against chronic inflammation and bacterial infection in ancient books, little research has been performed to investigate its anti-inflammatory effects and underlying mechanisms.

The present study investigated the effects of BM aqueous extracts on the production of inflammatory mediators in LPS-induced RAW264.7 cells. Meanwhile, the effects of BM

extracts on the cytokine secretion of GLM samples were also studied. To the best of our knowledge, the study demonstrated the anti-inflammatory effects of BM *in vitro* and *ex vivo* for the first time, and revealed the involvement of NF- κ B/MAPKs and chemokine (C-C motif) ligand (CCL)-5 signaling.

Materials and methods

Preparation and quality control of BM extracts. BM leaves (100 g) were cut into small pieces and immersed in distilled water. The mixture was treated with ultrasound for 1 h followed by heating at 100°C for 30 min twice. The supernatant was concentrated by rotary evaporation and kept at -80°C overnight. The freezing supernatant was then placed in a freeze dryer for 48 h to obtain the raw aqueous extract powder. The production ratio of BM was 12.3-13.6%. High-performance liquid chromatography (HPLC) analysis of BM aqueous extract was conducted on an Ultimate AQ-C18 column (250x4.6 mm; 5 μ m). The mobile phase consisted of acetonitrile (A) and water (B), using a gradient elution of 5-8% A at 0-5 min, 8-14% A at 5-30 min, 14-50% A at 30-55 min and 50-90% A at 55-60 min. The solvent flow rate was 1.0 ml/min and the column temperature was ambient. The detection wavelength was set at 250 nm. The berberine concentration in the extracts was 0.014-0.018 mg/ml [berberine was recommended as a representative compound in BM by Chinese Pharmacopoeia (13)] based on the absorbance at 250 nm assessed by ultraviolet-visible spectrophotometry (Fig. 1A). The powder was dissolved in phosphate-buffered solution (PBS) and passed through a 0.45- μ m filter for later use.

Cell culture. Mouse macrophage RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂. Human GLM samples were collected and cut into small pieces. The shredded tissues were filtered with 200- μ m nylon mesh and suspended in PBS solution. The filtered cells were centrifuged at 800 rpm for 5 min at room temperature and washed three times with PBS, and the precipitates were suspended and cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS (Gibco, Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin.

Cell proliferation assay. The effect of BM on cell proliferation was studied by cell growth assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) as described previously (14). Briefly, the cells were seeded onto a 96-well plate at a density of 4x10³ cells/well. Following serum starvation, different concentrations of BM (2.5, 5, 10, 20, 40, 60, 80 and 100 μ g/ml) were added to the wells, with 6 repeats for each concentration. LPS was added at 1 μ g/ml to simulate the inflammation status (15). The cell growth rate was detected using a cell proliferation MTT kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The purple formazan was dissolved with Dimethyl sulfoxide (Sigma, St. Louis, MO, USA) and detected at a wavelength of 540 nm. Triplicate independent experiments were performed.

Flow cytometry analysis. RAW264.7 cells (1×10^6 /well) were synchronized in G1 phase by serum deprivation, and different concentrations of BM (25, 50 and 100 $\mu\text{g/ml}$) and 1 $\mu\text{g/ml}$ LPS were then added. Cells were harvested after 48 h. Propidium iodide (PI)-stained single-cell suspension was analyzed on a FC5000 cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using CXP software version 2.0 (Beckman Coulter, Fullerton, CA, USA) for data analysis. The experiment was repeated three times. For apoptosis analysis, following BM treatment (25, 50 and 100 $\mu\text{g/ml}$) for 48 h, cells were stained with Annexin V and PI (Clontech Laboratories, Inc., Mountainview, CA, USA) for 15 min at room temperature. The apoptotic index was determined by flow cytometry with Flowjo software. 2',7'-Dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich; Merck KGaA) was used to measure ROS formation. After exposure to different concentrations of BM (25, 50 and 100 $\mu\text{g/ml}$) for 48 h, the cells were then incubated in DCFH-DA-containing medium (final concentration, 10 μM) at 37°C for 20 min. The cells were washed with PBS three times to remove DCFH-DA that had not entered into the cells. The intracellular concentration of DCFH-DA was then measured by flow cytometry.

NO detection. NO levels in the culture media were determined by the Griess Reagent system according to the manufacturer's protocols (Promega Corporation, Madison, WI, USA). Briefly, 50 μl cell culture supernatant was transferred into a 96-well plate and mixed with 100 μl Griess reagent (equal volumes of 1% w/v sulfanilamide in 5% v/v phosphoric acid and 0.1% w/v naphthylethylenediamine-HCl). Next, the plate was incubated for 5-10 min at room temperature, and absorbance was measured at 550 nm using a microplate reader. The amount of NO was calculated according to the standard sodium nitrite curve.

Western blot analysis. Cells were lysed in RIPA buffer (Sigma) containing a protease inhibitor mixture (Roche Diagnostics, GmbH, Mannheim, Germany). The protein concentration was determined with the bicinchoninic acid assay (Thermo Fisher Scientific, Inc., Bonn, Germany). Quantified protein lysates (15 μg) were resolved by 10% sodium dodecyl sulfate - 12% polyacrylamide gel electrophoresis. The proteins were then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Each membrane was blocked for 2 h with 2% bovine serum albumin (Sigma) and then incubated overnight at 4°C with 1 $\mu\text{g/ml}$ primary antibody (dilution, 1:1,000). The antibodies COX-2 (A5787), NF- κB (A2574), p-NF- κB (AP0475), I κB kinase (IKK) α (A2062), IKK β (A2087), ERK (A0229), p-ERK (AP0472), JNK (A6117), p-JNK (AP0276), p38 (A0227), p-p38 (AP0056) were obtained from ABclonal (Boston, MA, USA). The antibodies p-IKK α/β (2697), IKK α (4814) p-IKK α (2859) and β -actin (4970s) were purchased from Cell Signaling Technology (Danvers, MA, USA). iNOS (bs-0162R) was provided by BIOSS (Beijing, China). After 3 washes with Tris-buffered saline with 0.05% Tween-20, the membranes were incubated with HRP-conjugated anti-rabbit (7074s) or anti-mouse (7076) secondary antibodies (dilution, 1:2,000; Cell Signaling Technology) for 2 h at room temperature. The signals were visualized using the ECL Advance reagent (GE Healthcare) and quantified using

Table I. Primer sequence for target genes.

Gene (<i>Mus musculus</i>)	Primer sequence (5'-3')
iNOS	F: GCTACCAAACCTGGATATAATCAGGA R: CCAGGTAGCTATGGTACTCCAGAA
COX-2	F: GCTACCAAACCTGGATATAATCAGGA R: CCAGGTAGCTATGGTACTCCAGAA
IL-1 β	F: GCTACCAAACCTGGATATAATCAGGA R: CCAGGTAGCTATGGTACTCCAGAA
IL-6	F: GCTACCAAACCTGGATATAATCAGGA R: CCAGGTAGCTATGGTACTCCAGAA
CCL-2	F: CATCCACGTGTTGGCTCA R: GATCATCTTGCTGGTGAATGAGT
CCL-3	F: TGCCCTTGCTGTTCTTCTCT R: GTGGAATCTTCCGGCTGTAG
CCL-5	F: TGCAGAGGACTCTGAGACAGC R: GAGTGGTGTCCGAGCCATA
sTNFR1	F: TCTTCTCATTCTGCTTGTGG R: GGTCTGGGCCATAGAACTGA
GAPDH	F: GGGTTCCTATAAATACGGACTGC R: CCATTTTGTCTACGGGACGA

iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; IL, interleukin; chemokine (C-C motif) ligand; sTNFR1, secreted tumor necrosis factor receptor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Quantity One software (version 4.5; Bio-Rad Laboratories, Hercules, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from the cells was extracted using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and RT was performed using a first strand cDNA synthesis kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocols. PCR amplification conditions consisted of an initial denaturation step at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 45 sec; a final extension period at 72°C for 5 min completed the amplification. qPCR analysis was performed using a SYBR-Green kit (Roche Diagnostics, Basel, Switzerland) on a Roche lightcycler 480 detector (Roche Diagnostics, GmbH, Mannheim, Germany). The primers for iNOS, COX-2, IL-1 β , IL-6, CCL-2, CCL-3, CCL-5, secreted tumor necrosis factor receptor 1 (sTNFR1) and glyceraldehyde 3-phosphate dehydrogenase are listed in Table I. Cq value was measured during the exponential amplification phase. The relative expression level (defined as fold-change) of the target gene was given by $2^{-\Delta\Delta Cq}$ and normalized to the internal control.

Luciferase reporter analysis. The pathway reporter plasmids of phosphorylated (p)NF- κB -Luc and phosphorylated activated protein 1 (pAP-1)-Luc, which contain NF- κB or AP-1 binding sites in the promoter region, were purchased from Clontech Laboratories, Inc. Overnight cultured RAW264.7 cells in 96-well plates were transfected with 100 ng

pNF- κ B-Luc or pAP-1-Luc reporter vector and 5 ng phRL-TK *Renilla* luciferase reporter (Promega Corporation) using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). At 16 h post-transfection, the cells were treated with various concentrations of BM and LPS (for NF- κ B reporter). After another 24 h, the cells were lysed for Firefly/*Renilla* luciferase activity assay using a dual-luciferase assay kit (Promega Corporation). The relative luciferase activities were normalized by LPS/IFN- γ or PMA control.

Immunofluorescence analysis. RAW264.7 cells (3×10^5 /well) were seeded in 24-well plates containing coverslips. Following LPS or BM treatment for 24 h, coverslips were then fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100. After blocking in 10% goat serum (DAKO, Glostrup, Denmark) for 1 h at room temperature, the coverslips were incubated with primary NF- κ B antibody (1:100; A2574; ABclonal) overnight at 4°C and then with secondary Alexa Fluor 555-conjugated antibody (1:200; 4413s; Cell Signaling Technology) for 2 h at room temperature. Finally, the samples were incubated with 1 μ g/ml 4',6-diamidino-2-phenylindole DAPI (Sigma) for 15 min at room temperature for nuclear staining and detected under a confocal microscope (LSM710; Carl Zeiss, Jena, Germany).

Cytokine array. Mouse and human inflammatory antibody array C1 kits were purchased from RayBiotech (Norcross, GA, USA). Briefly, cell supernatants from RAW264.7 cells or primary cultured GLM samples prior to and subsequent to BM treatment were collected. Once the antibody array membranes had been blocked in 5% BSA for 30 min at room temperature, cell supernatants were cultured with antibody arrays overnight at 4°C and washed three times. A biotinylated antibody cocktail was then incubated with the membranes for 2 h, followed by signal amplification with horseradish peroxidase-streptavidin. Finally, the signals were detected by chemiluminescence method with an ECL kit purchased from GE Healthcare Life Sciences (Little Chalfont, UK).

Ex vivo human GLM sample experiments. All human GLM patients provided written informed consent for the use of clinical specimens for medical research. Studies using human tissue were reviewed and approved by the committee for the ethical review of research involving human subjects of Guangzhou University of Chinese Medicine (Guangzhou, Guangdong, China). The methods for the collection and application of human specimens were in accordance with International Ethical Guidelines for Biomedical Research Involving Human Subjects. Clinical specimens were obtained based on the diagnosis of clinical syndromes, ultrasound findings and pathological characteristics. A total of 10 GLM specimens were sliced into small pieces and put into digestion buffers (StemCell Technologies, Vancouver, Canada) containing collagenase and hyaluronidase for 2-3 h with agitation. The cell suspension was then filtered through a 100- μ m cell strainer and the filtered content was then centrifuged at 200 \times g for 8 min at 4°C. The cell pellets were then resuspended in 1 ml RPMI-1640 medium above 5 ml 45% Percoll (GE Healthcare, Uppsala, Sweden) in the middle and 5 ml 60% Percoll at the bottom in a 15-ml tube. Mononuclear cells were collected from the cell layer in the

interphase between 45 and 60% Percoll. The cells were then cultured in RPMI-1640 medium at 37°C and treated with BM. The cells supernatants were then collected for the cytokine array detection and the cellular protein lysates were subjected to western blot assay as stated above.

Statistical analysis. Data are presented as the mean \pm standard error of the mean (SEM). The results were analyzed by SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Mean values were derived from at least three independent experiments. Statistical comparisons between experimental groups were performed by one-way analysis of variance followed by post hoc Dunnett's test. The level of statistical significance was set at $P < 0.05$.

Results

Preparation and LC-mass spectrometry (MS) analysis of BM. As with the majority of TCM formulae, a decoction of BM used clinically. The pharmacological activity of BM and the repeatability of the experiments performed using it are dependent on the controlled quality of the decoction. In the present study, in order to maintain a consistent quality, BM was extracted with water and prepared carefully. The preparation procedure is summarized in Fig. 1B. HPLC analysis further validated that the chemical fingerprints were in accordance in three different batches (Fig. 1A). Meanwhile, quality analysis of BM was performed with LC-MS. As shown in Fig. 1C, the MS spectra revealed the presence of chlorogenic acid, jateorhizine, magnoflorine and berberine in the BM extract.

BM exhibits few inhibitory effects on RAW264.7 proliferation, cell cycle progression and apoptosis induction. To study whether BM could inhibit the proliferation of RAW264.7 cells, LPS was added as positive inducer of inflammation and three batches of BM were tested in a dose- and time-dependent manner, respectively. The results showed that BM exerted few inhibitory effects on the proliferation of RAW264.7 cells, which not only validated the bioactivity consistency between three different batches of BM, but also implied that BM had few cytotoxic effects (Fig. 2A). Similarly, cell cycle analysis also indicated that BM had limited influence on the distribution of the cell cycle (Fig. 2B). Meanwhile, Annexin V-FITC/PI assay demonstrated that following BM administration, limited changes in the apoptotic ratio of RAW264.7 cells occurred (Fig. 2C). All these results indicated that the anti-inflammation activity of BM was not due to its survival and proliferation inhibitory effects.

BM inhibits ROS generation and NO synthesis. During inflammation, a significant increase in oxygen consumption would result in a massive elevation of intracellular ROS, which acts as an upstream signal to promote the inflammation process and even destroys cells to induce abscess formation (16). To determine whether the LPS-induced intracellular redox state could be suppressed by BM, ROS production was detected prior to and following BM treatment. The results showed that the level of ROS in response to LPS was significantly higher than that of unstimulated cells. However, with the increasing dose of BM in the

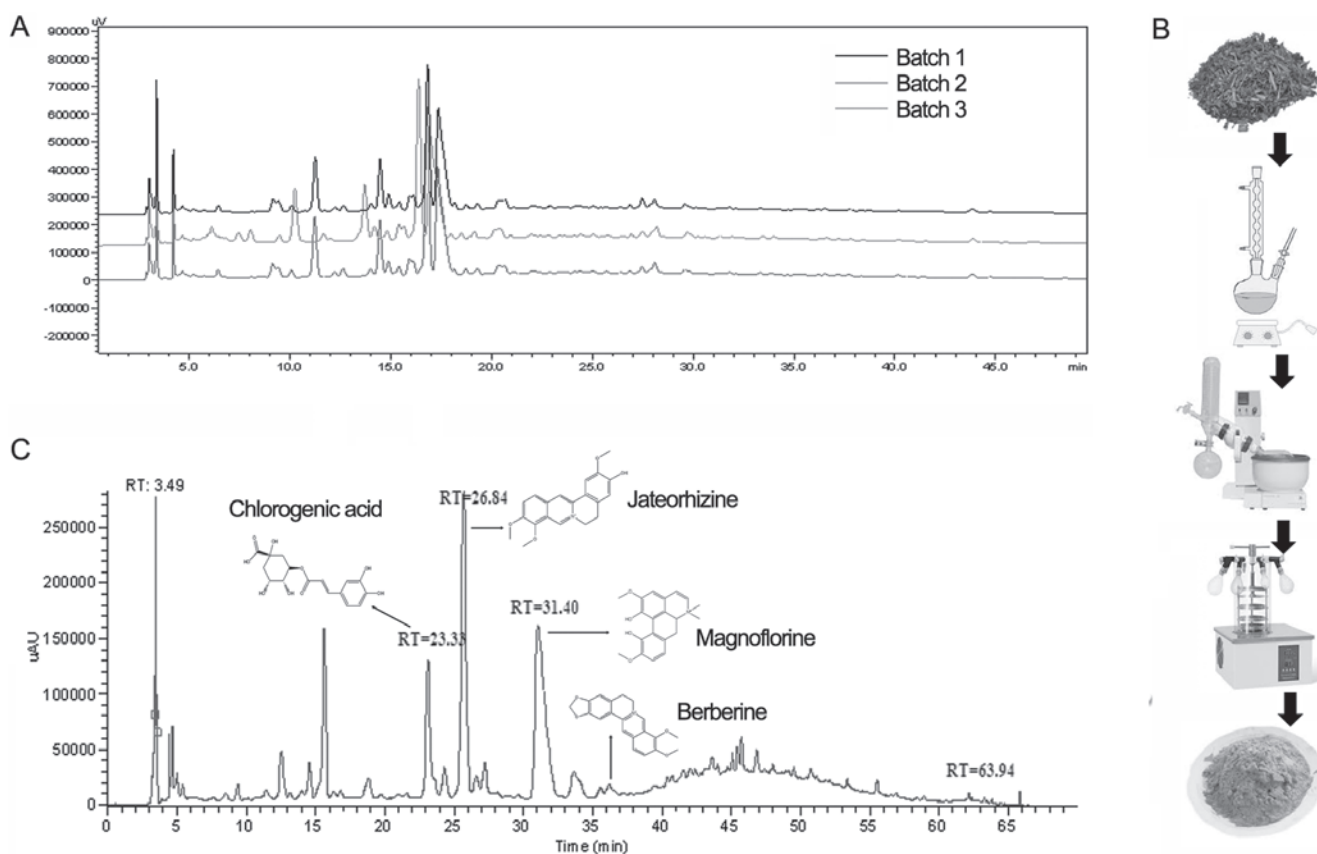


Figure 1. Preparation and quality control of BM extracts. (A) Chemical fingerprints of three different batches of BM extracts detected by high-performance liquid chromatography. (B) Preparation image flow chart of BM extracts. BM extract was prepared by routine boiling and concentrated by rotary evaporation and freeze dried for later use. (C) Liquid chromatography-mass spectrometry identified three major chemicals in the BM extracts: Chlorogenic acid, jateorhizine and magnoflorine. BM, broadleaf *Mahonia*.

medium, the ROS production was significantly inhibited in a dose-dependent manner (Fig. 3A and B). Furthermore, since excessive NO production is a critical marker of inflammatory diseases, such as rheumatoid arthritis and autoimmune diseases, the present study detected whether BM also led to a decrease in NO levels. RAW264.7 cells were treated with different doses of BM for 2 h prior to stimulation with LPS (1 $\mu\text{g}/\text{ml}$) (17). NO production is reflected in the accumulation of nitrite in the cell culture medium. The results revealed that LPS stimulation caused a significant increase of nitrite concentration, while BM administration inhibited NO release in a dose-dependent manner, with >50% inhibition at a concentration of 100 $\mu\text{g}/\text{ml}$ (Fig. 3C). These results suggested that BM was capable of scavenging ROS level and thereby alleviating inflammation severity.

BM inhibits iNOS/COX-2 expression and pro-inflammatory cytokine secretion. The iNOS gene is known to be the primary regulator of NO production in macrophages, and the COX-2 gene is usually increased under inflammation and malignant conditions (18). To determine the anti-inflammatory effects of BM, the expression levels of iNOS and COX-2 were measured by western blotting and qPCR analysis. As shown in Fig. 4A, the protein expression levels of iNOS and COX-2 were significantly elevated following LPS stimulation, but the two proteins displayed a marked downregulated tendency

in response to increasing concentration of BM treatment. Consistently, the mRNA expression levels of iNOS and COX-2 were also suppressed by BM (Fig. 4B). Since NF- κB acts as the common transcription factor of the two genes, the results indicated that BM may inhibit inflammation via suppressing NF- κB signaling.

During the progression of inflammation, IL-1 β and IL-6 are known to be pro-inflammatory cytokines, which are constitutively secreted and activated, mediating macrophage chemotaxis, angiogenesis and sustained tissue destruction (19,20). qPCR analysis was also applied to determine the levels of IL-1 β and IL-6 prior to and following BM administration. When the cells were induced by LPS, the expression levels of IL-1 β and IL-6 mRNA were markedly increased. However, when BM was added to the media, the mRNA levels of the two cytokines were significantly decreased in a dose-dependent manner (Fig. 4C). All these results indicated that BM is able to block the secretion of pro-inflammatory cytokines.

BM blocks the transcriptional activities of NF- κB and AP-1. Since NF- κB and AP-1 control the transcription of iNOS/COX-2 and a variety of pro-inflammatory cytokines, the present study investigated the effect of BM on these signaling pathways using a luciferase reporter assay. As demonstrated in Fig. 5, the transcriptional activities of NF- κB and AP-1 were inhibited dose-dependently following BM treatment, but the

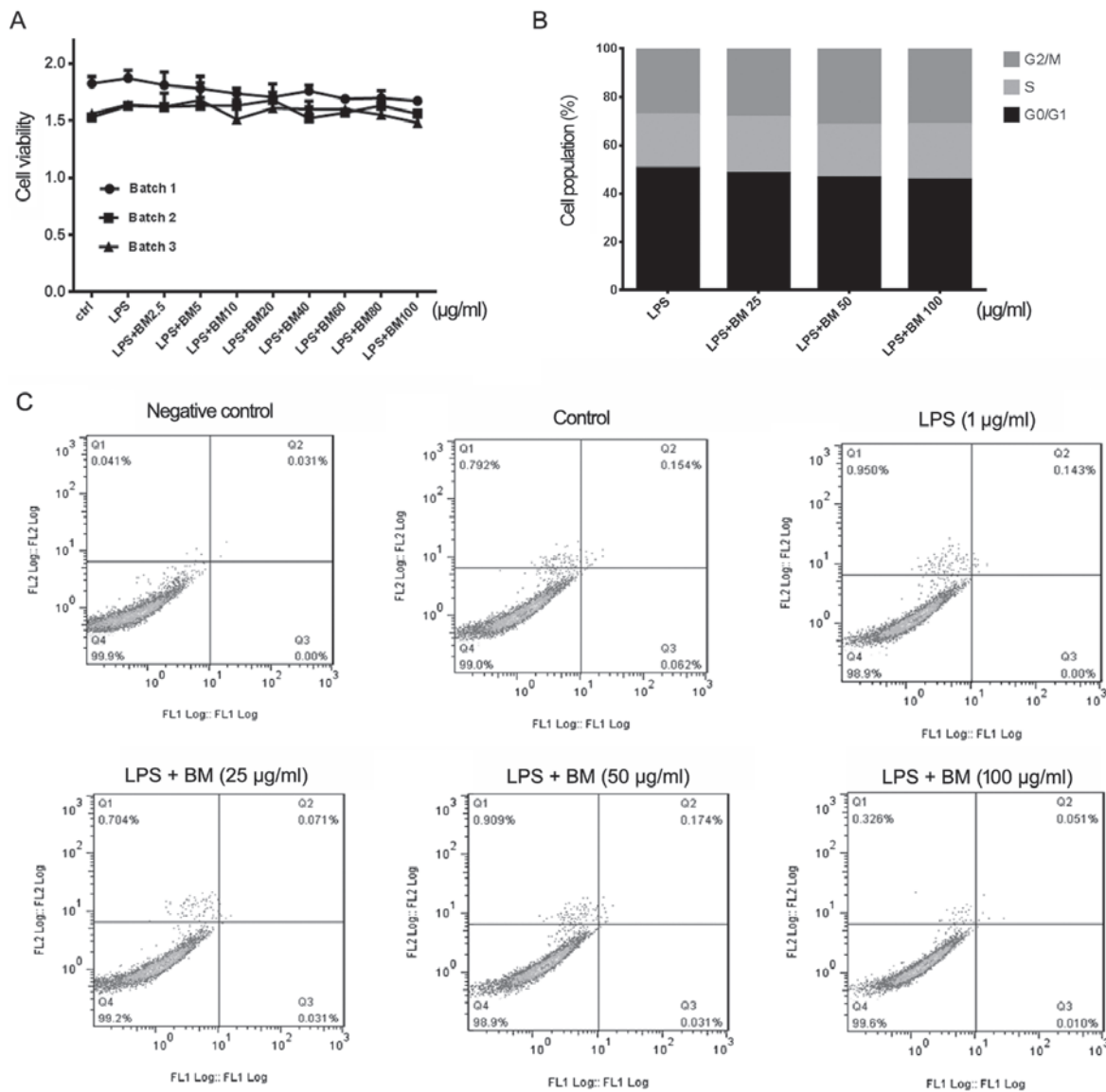


Figure 2. Effects of BM extracts on the proliferation of RAW264.7 cells. (A) MTT assay revealed that BM had limited effect on the proliferation of RAW264.7 cells. (B) Flow cytometry analysis revealed that BM exhibited limited effect on the cell cycle distribution of RAW264.7 cells. (C) Annexin V/propidium iodide analysis revealed that BM could not induce apoptosis in RAW264.7 cells. BM, broadleaf *Mahonia*; LPS, lipopolysaccharide.

inhibition ratio of NF- κ B was higher. These results indicated that BM blocked LPS-induced NF- κ B and AP-1 signaling, leading to the inhibition of LPS-induced production of NO and COX-2.

BM suppresses LPS-induced activation of NF- κ B and MAPK signaling. Considering the nuclear translocation of the p65 subunit is a critical step determining its downstream transcriptional activity, the present study investigated whether BM affects the subcellular compartmentalization of p65. RAW264.7 cells were pretreated with BM for 12 h and stimulated with LPS for another 1 h, and the intracellular p65 distribution was then evaluated by confocal microscopy. As shown in Fig. 6A, the p65 subunit was mainly distributed in the nucleus following BM treatment. However, pre-treatment with BM significantly reduced the p65 translocation compared with that in the control cells with LPS stimulation. In order to identify the upstream target of BM-mediated NF- κ B

suppression, the expression status of IKK and I κ B α was investigated. RAW264.7 cells were stimulated with LPS for 1 h in the presence or absence of BM. As shown in Fig. 6B-D, the phosphorylated level of IKK α / β was significantly elevated following LPS stimulation, but began to decrease gradually with the increasing dose of BM. However, the expression status of IKK α and IKK β exhibited limited changes in response to BM treatment. Notably, the phosphorylation of I κ B α and p65 was also inhibited following BM administration in the presence of LPS stimulation, respectively. All these results indicated that BM inhibited NF- κ B activation by blocking the phosphorylation of I κ B α and its upstream kinase IKK α / β .

Since the MAPK pathway is also involved in regulating NF- κ B and AP-1 signaling transduction, the phosphorylated levels of ERK1/2, JNK and p38 prior to and following BM treatment were evaluated. As expected, LPS significantly induced the phosphorylation of all three after 1 h of incubation. Notably, BM inhibited the phosphorylation of JNK and

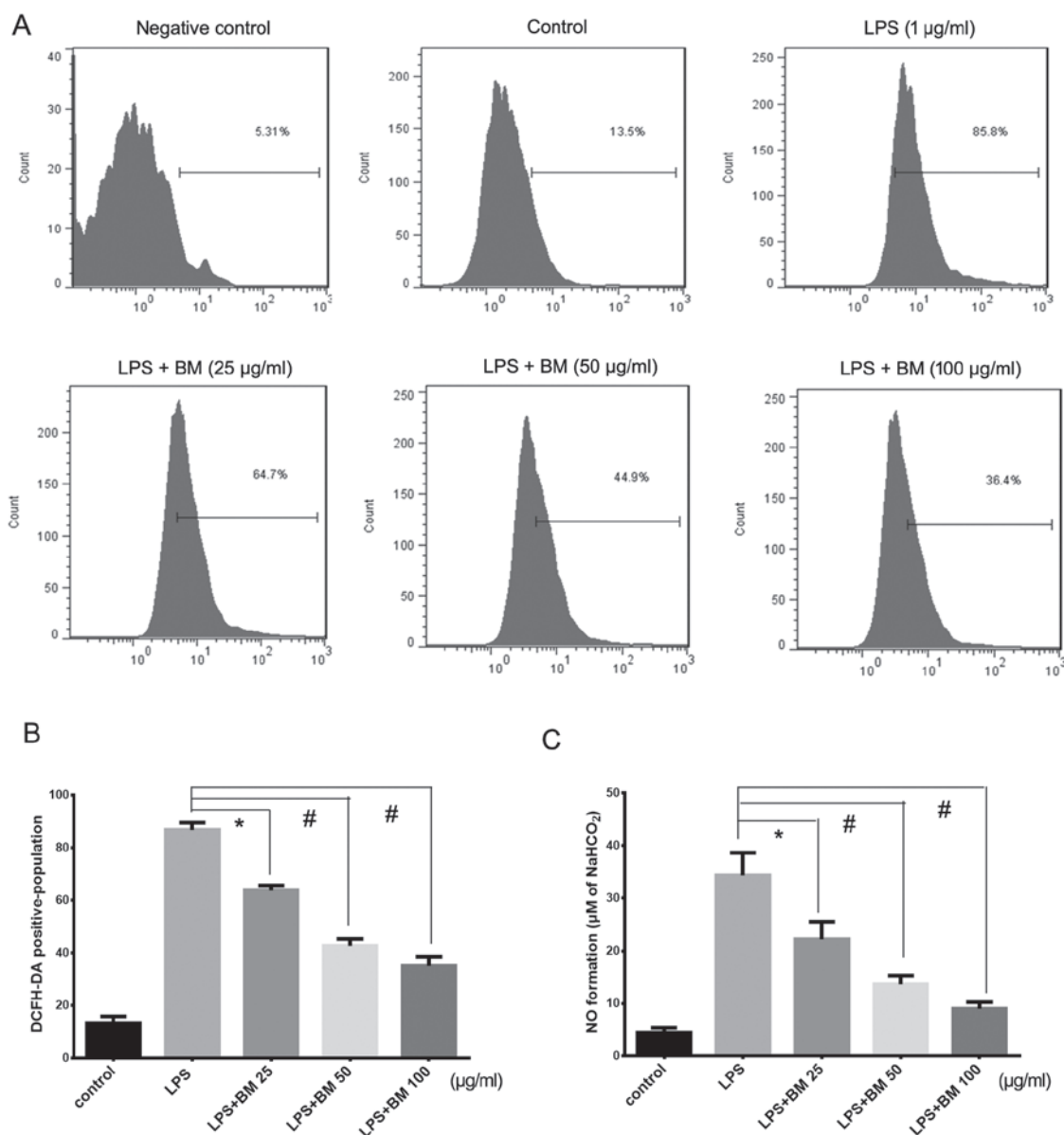


Figure 3. Effects of BM extracts on ROS generation. (A and B) DCFH-DA analysis showed that BM inhibited ROS level induced by lipopolysaccharide (LPS) stimulation. (C) NO formation analysis demonstrated that BM could dose-dependently inhibit NO synthesis in RAW264.7 cells induced by LPS (* $P < 0.05$ and # $P < 0.01$ vs. LPS group). BM, broadleaf *Mahonia*; LPS, lipopolysaccharide ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescein diacetate; NO, nitric oxide.

p38 in a dose-dependent manner, but had little effect on the phosphorylation status of ERK1/2 (Fig. 6C and D). These findings suggested the ability of BM to suppress JNK and p38 activation, which may be responsible for the inhibition of NF- κ B signaling and pro-inflammatory cytokine expression.

Identification of CCL-5 as the main target of BM in cell and GLM samples. Although it was demonstrated that BM inhibited the inflammation via the NF- κ B and MAPK pathways, the precise molecular mechanisms accounting for its anti-inflammatory effects remained unclear. In order to determine the molecular target of BM in RAW264.7 cells, an inflammation array was applied. The supernatants of RAW264.7 cells prior to and following BM treatment were collected and subjected to array test. As shown in Fig. 7A, a total of 40 inflammatory-related cytokines were detected. Following BM administration, the expression levels of CCL-2

(also known as monocyte chemoattractant protein-1), CCL-3 (also known as macrophage inflammatory protein-1 α), CCL-5 (also known as regulated on activation, normal T cell expressed and secreted) and sTNFR1 were significantly inhibited. The results indicated that BM could inhibit the inflammation via interacting with multiple cytokines. qPCR further validated that among the 4 cytokines, the inhibited ratios of CCL-3 and CCL-5 were the most significant, implying that the anti-inflammatory molecular target of BM may be attributed to their downregulation (Fig. 7B). Importantly, western blotting results further validated that the inhibitory effects of BM on p65 phosphorylation were relieved after adding CCL-5, indicating that the anti-inflammatory effects of BM may be partly attributed to CCL-5 suppression (Fig. 7C).

Meanwhile, human GLM samples were also collected to validate the molecular mechanisms of BM (Fig. 8A). Consistent with the *in vitro* findings, cytokine array results

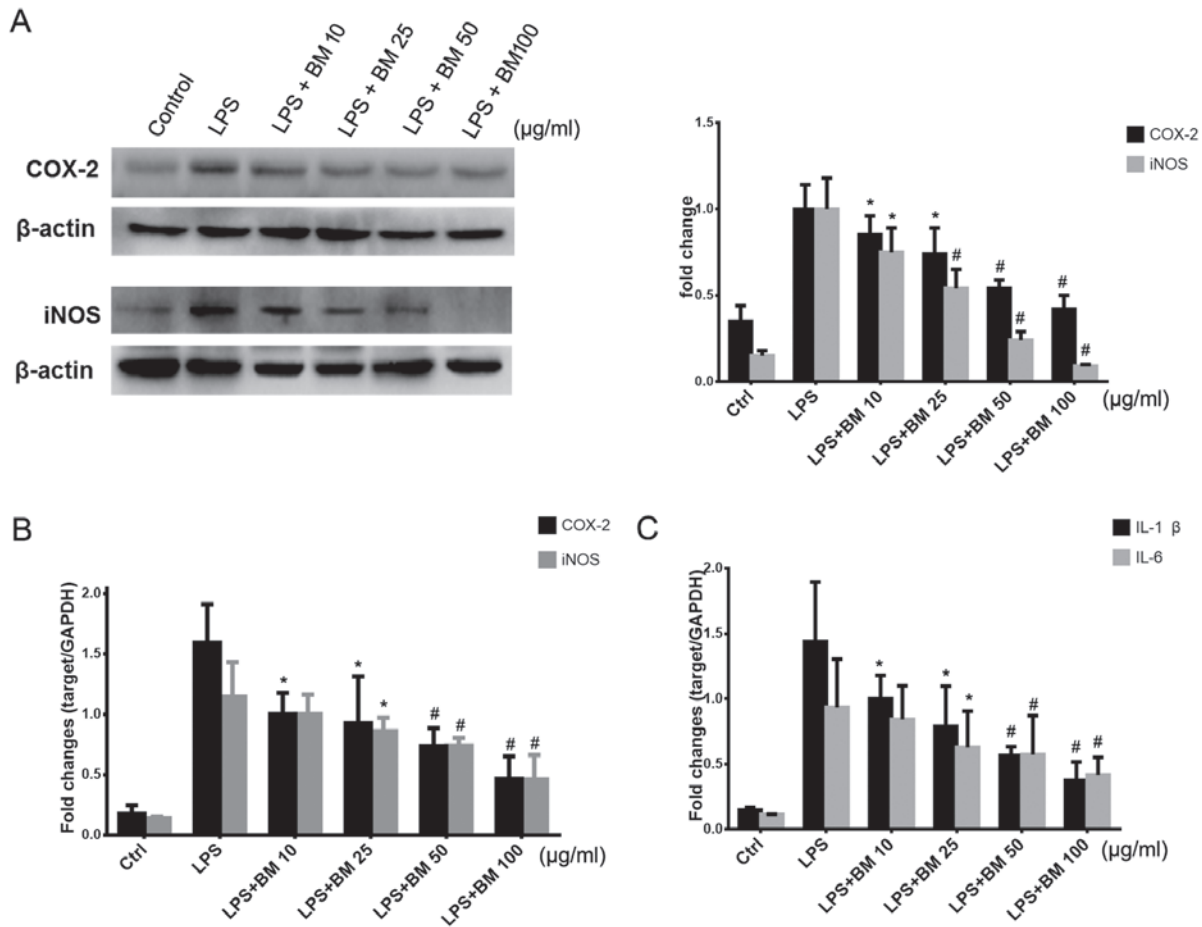


Figure 4. Inhibitory effects of BM on inflammation. (A) BM significantly inhibited the expression of COX-2 and iNOS in a dose-dependent manner. (B and C) Quantitative polymerase chain reaction results revealed that BM was capable of suppressing the mRNA expression of COX-2, iNOS, IL-1 β and IL-6 (* P <0.05 and # P <0.01 vs. LPS group). BM, broadleaf *Mahonia*; LPS, lipopolysaccharide; ROS, reactive oxygen species; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; IL, interleukin.

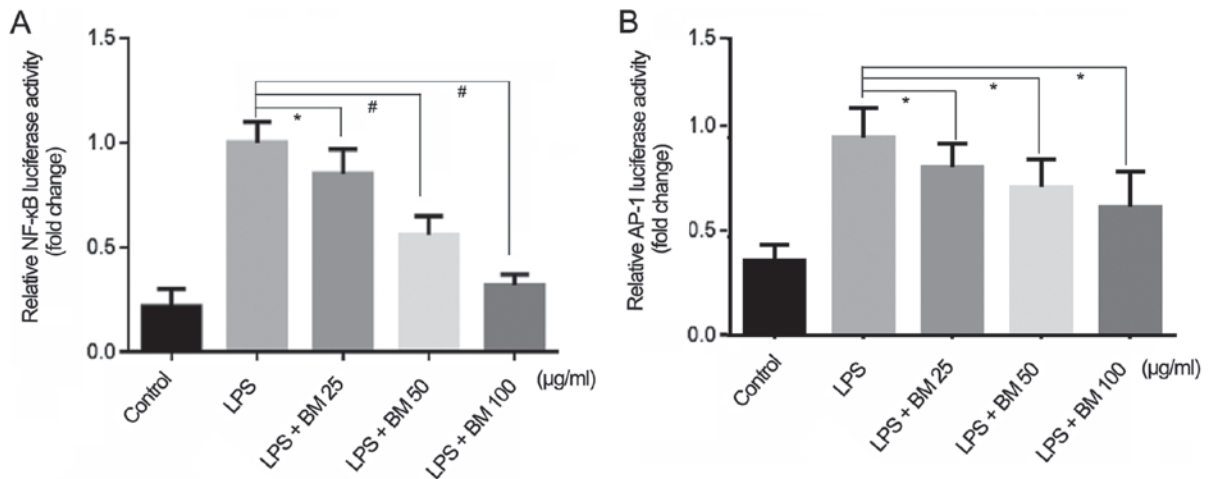


Figure 5. Luciferase reporter analysis showed that BM could dose-dependently inhibit the transcriptional activity of NF- κ B and AP-1 (* P <0.05 and # P <0.01 vs. LPS group). BM, broadleaf *Mahonia*; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; AP-1, activator protein 1.

indicated that the expression of CCL-2, CCL-3, CCL-5 and sTNFR was inhibited following BM treatment; the results further confirmed the anti-inflammatory effects of BM *ex vivo* (Fig. 8B and Table II). Similar to the results in *in vitro* studies, CCL-5 also exhibited the highest inhibition ratio

following BM treatment, and western blotting results also indicated that the expression of phosphorylated p65, iNOS and COX-2 was inhibited by BM on GLM samples (Fig. 8C). All these results indicated that BM could inhibit inflammation via suppressing CCL-5 in *in vitro* and *ex vivo* models.

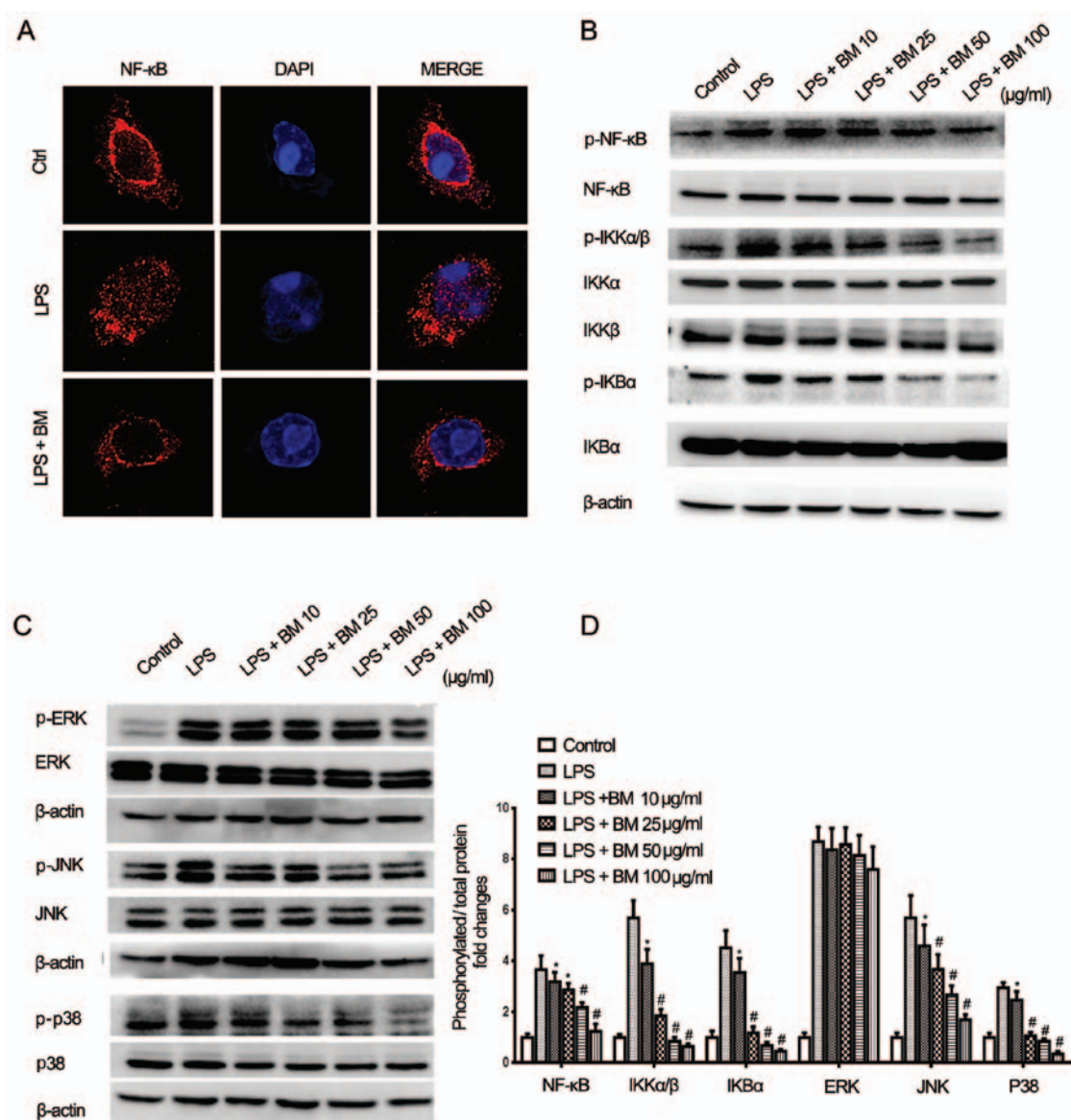


Figure 6. BM inhibits the NF- κ B and MAPK pathways. (A) Confocal fluorescence microscopy showed that BM could effectively block the nuclear translocation of NF- κ B induced by LPS. (B and C) BM inhibited the NF- κ B and MAPK pathways, presenting as the downregulation of phosphorylated NF- κ B, IKK α / β , IKB α , JNK and p38. (D) Relative quantification of phosphorylated/total proteins, including NF- κ B, IKK α / β , IKB α , ERK, JNK and p38 ($^*P<0.05$ and $^{\#}P<0.01$ vs. LPS group). BM, broadleaf *Mahonia*; LPS, lipopolysaccharide; p-, phosphorylated; NF- κ B, nuclear factor- κ B; MAPK, mitogen-activated phosphate kinase; JNK, c-Jun N-terminal kinase; IKK, I κ B kinase; ERK, extracellular signal-regulated kinase.

Discussion

Chronic inflammation is a complex set of interactions involving multiple cells and cytokines that can arise in any tissue in response to traumatic, post-ischemic, infectious, autoimmune or toxic injuries (21). GLM is considered to be an autoimmune reaction to the materials secreted from the mammary ducts, and granuloma formation is the most representative syndrome of the disease. Since macrophages are the first line of host defense during the initiation of the innate and adaptive immune responses and are hypothesized to be the root inducing granuloma occurrence (22), it is logical to develop anti-GLM drugs in the perspective of macrophages.

Macrophage activation could result in the secretion of various pro-inflammatory mediators, including IL-1 β , IL-6, COX-2 and iNOS (23). Deregulated overproduction of IL-1 β

and IL-6 has been demonstrated to serve pathological roles in chronic inflammatory diseases, including Castleman's disease, Crohn's disease, rheumatoid arthritis and juvenile idiopathic arthritis (24,25). Humanized anti-IL-1 β or -IL-6 antibodies were also developed into therapeutic agents, such as tocilizumab and canakinumab, for these diseases, and exhibited outstanding anti-inflammatory effects in clinical trials (26). Therefore, IL-1 β or IL-6 were selected as parameters to investigate the anti-inflammatory effects of BM in the present study. It was demonstrated that following BM treatment, the mRNA expression levels of IL-1 β and IL-6 were downregulated, implying that macrophage activity and the pro-inflammation process may be inhibited. In particular, inhibition of IL-1 β expression by BM was more enhanced than that of IL-6, indicating the potential of BM to treat typical inflammation-related disorders.

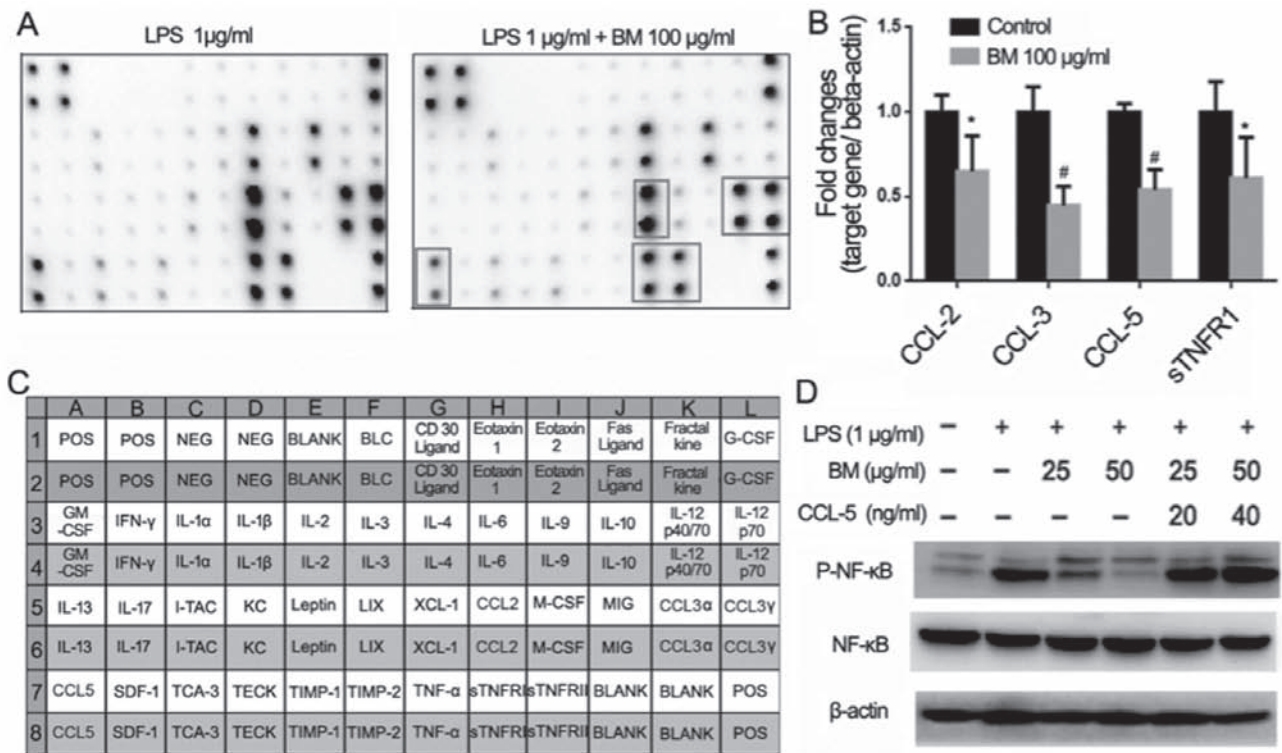


Figure 7. Effects of BM on inflammation-related cytokine expression. (A) Cytokine array analysis revealed that BM inhibited the expression of CCL-2, CCL-3, CCL-5 and sTNFR1. (B) Quantitative polymerase chain reaction validated that among the four cytokines, BM exhibited the most significant inhibitory effects on both CCL-3 and CCL-5 ($P < 0.05$ and $^{\#}P < 0.01$ vs. control group). (C and D) The inhibitory effects of BM on NF- κ B phosphorylation were recovered after adding CCL-5, implying that CCL-5 may be the main target of BM. BM, broadleaf *Mahonia*; LPS, lipopolysaccharide; p-, phosphorylated; NF- κ B, nuclear factor- κ B; IL, interleukin; sTNFR1, secreted tumor necrosis factor receptor 1; CCL, chemokine (C-C motif) ligand; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN- γ , interferon gamma; IL, interleukin; I-TAC, interferon-inducible T-cell alpha chemoattractant; KC, chemokine (C-X-C motif) ligand 1; LIX, C-X-C motif chemokine 5; XCL-1, chemokine (C motif) ligand; M-CSF, macrophage colony-stimulating factor; MIG, chemokine (C-X-C motif) ligand 9; SDF1, stromal cell-derived factor 1; TCA-3, chemokine (C-C motif) ligand 1; TECK, chemokine (C-C motif) ligand 25; TIMP, tissue inhibitor of metalloproteinases; TNF- α , tumor necrosis factor α ; sTNFR, soluble tumor necrosis factor receptors; ICAM, intercellular Adhesion Molecule 1; PDGF, platelet-derived growth factor.

Meanwhile, BM also resulted in a significant reduction of iNOS and COX-2 expression. Of the two isoforms of COX, COX-1 is suggested to provide a physiological level of prostaglandins for normal platelet, stomach and kidney function, whereas COX-2 is highly induced at inflammatory sites in animals, as well as in patients with inflammatory diseases (27). Since COX-2 is responsible for prostaglandin E2 production, a critical cytokine considered as one of the strongest inflammatory mediators, COX-2 is usually believed to be a therapeutic target for inflammatory diseases, and COX-2 inhibitors, such as celecoxib, are widely applied to fight against chronic inflammation clinically (28,29). The present data showed that BM significantly inhibited COX-2 protein expression in a dose-dependent manner, suggesting that the anti-inflammatory effect of BM is closely associated with its inhibitory effect on COX-2. Additionally, since iNOS is responsible for NO synthesis, which significantly contributes to the initiation and perpetuation of numerous inflammatory responses (30,31), the NO concentration in the cell supernatants was detected following BM treatment. The dose-dependent reduction of NO concentration by BM was consistent with the decreased expression of iNOS, indicating that the blocking effect of BM on NO formation may be attributed to the suppressed iNOS expression. Actually, ROS and reactive nitrogen oxide species (RNOS), including NO, are

oxidants elevated in the inflammatory process. High levels of ROS and RNOS are capable of damaging inflammatory cells and tissues, and thereby promoting abscess formation. ROS could also act as upstream signals to trigger pro-inflammatory cytokine expression, and ROS scavenging is considered to be a novel therapeutic strategy to avoid inflammatory-associated tissue damage (32,33). The present results demonstrated that BM was capable of reducing LPS-induced ROS accumulation in RAW264.7 cells, indicating that BM may be active in alleviating ROS-induced damage during GLM development.

NF- κ B and MAPK pathways have been shown to act as common downstream signaling pathways in mediating the inflammatory responses of various types of tissues (8,9,34). The most predominantly characterized NF- κ B complex is a p50/p65 heterodimer, which is kept inactive in the cytoplasm of resting cells by binding with the inhibitory subunit of I κ B α (35,36). Upon stimulation, I κ B α is phosphorylated by IKK thereby releasing NF- κ B, which subsequently translocates into the nucleus and activates downstream gene expression, including that of IL-1 β , IL-6, COX-2 and iNOS (37). The present results indicated that BM extract significantly inhibited NF- κ B activation, presenting as suppressed transcriptional activity, and decreased expression of p-p65, I κ B α and IKK. Additionally, confocal imaging analysis also demonstrated that the nuclear transport of p65 was also blocked following BM administration.

Table II. Human inflammation cytokine array.

No.	A	B	C	D	E	F	G	H	I	J	K	M
1	POS	POS	NEG	NEG	Eotaxin-1	Eotaxin-2	GCSF	GM-CSF	ICAM-1	IFN- γ	I-309	IL-1 α
2												
3	IL-1	IL-2	IL-3	IL-4	IL-6	IL-6R	IL-7	IL-8	IL-10	IL-11	IL-12 P40	IL-12 P70
4												
5	IL-13	IL-15	IL-16	IL-17A	IP-10	CCL2	MCP-2	M-CSF	MIG	CCL3 α	CCL3 β	MIP-1 δ
6												
7	CCL5	TGF- β 1	TNF- α	TNF- β	sTNF R1	sTNF RII	PDGF-BB	TIMP-2	Blank	Blank	NEG	POS
8												

IL, interleukin; IFN- γ , interferon- γ ; MIP-1 α , macrophage inflammatory protein-1 α ; TNF- α , tumor necrosis factor- α ; TGF- β 1, transforming growth factor- β 1; RANTES, regulated on activation, normal T cell expressed and secreted; GCSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN- γ , interferon gamma; IL, interleukin; I-TAC, interferon-inducible T-cell alpha chemoattractant; KC, chemokine (C-X-C motif) ligand 1; LIX, C-X-C motif chemokine 5; XCL-1, chemokine (C motif) ligand; CCL2, chemokine (C-C motif) ligand 2; M-CSF, macrophage colony-stimulating factor; MIG, chemokine (C-X-C motif) ligand 9; CCL3, chemokine (C-C motif) ligand 3; CCL5, chemokine (C-C motif) ligand 5; SDF1, stromal cell-derived factor 1; TCA-3: chemokine (C-C motif) ligand 1; TECK, chemokine (C-C motif) ligand 25; TIMP, tissue inhibitor of metalloproteinases; TNF- α , tumor necrosis factor α ; sTNFR, soluble tumor necrosis factor receptors; ICAM, intercellular Adhesion Molecule 1; PDGF, platelet-derived growth factor.

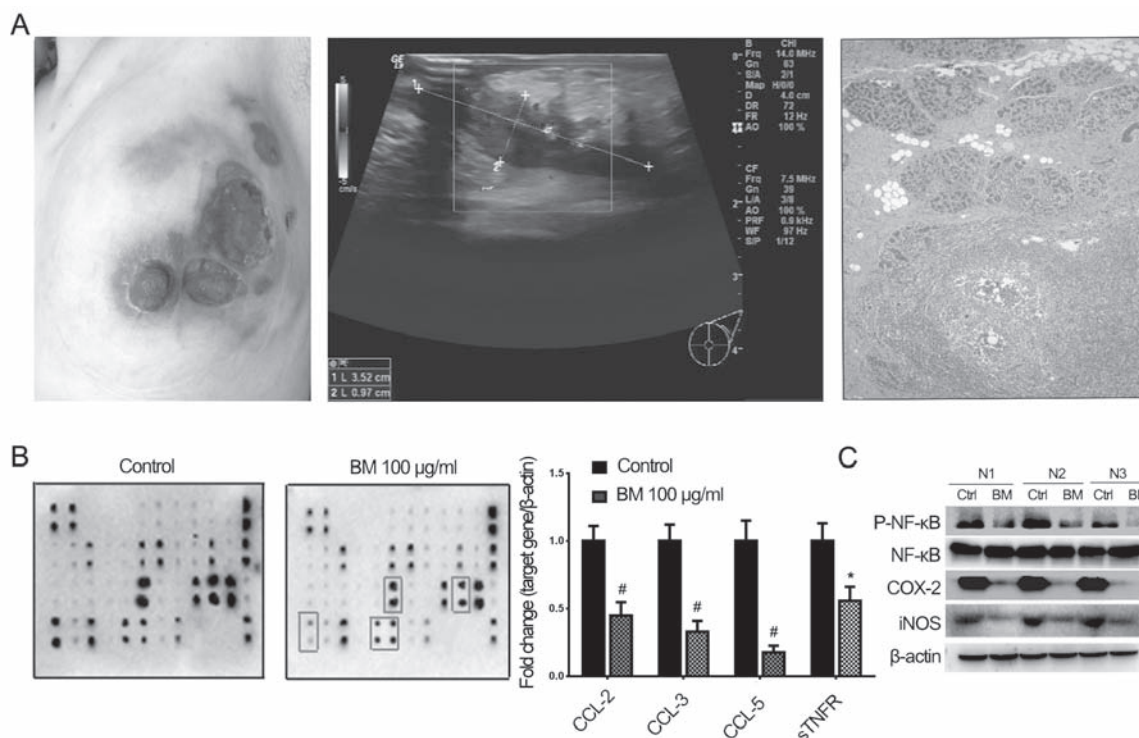


Figure 8. Inflammation inhibition effects of BM on clinical GLM samples. (A) Representative pictures of GLM including mammary symptoms, ultrasound image and pathological diagnosis. (B) Cytokine array and quantitative polymerase chain reaction analysis revealed that following BM treatment, the expression of CCL-3, CCL-5 and sTNFR1 was inhibited (* P <0.05 and # P <0.01 vs. control group). (C) Western blotting results validated that BM exhibited inhibitory effects on the expression of p-NF- κ B, COX-2 and iNOS. BM, broadleaf *Mahonia*; GLM, granulomatous lobular mastitis; NF- κ B, nuclear factor- κ B; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; CCL, chemokine (C-C motif) ligand.

All these results implied that NF- κ B suppression may be mainly responsible for the anti-inflammatory effects of BM. MAPKs are also involved in the progress of inflammation. Previous studies have demonstrated that the inflammatory process is strongly blocked by inhibiting MAPK family members, including p38, JNK and ERK (38,39). Meanwhile, MAPKs and NF- κ B can collaborate synergistically to induce pro-inflammatory cytokine gene products and releases (40,41).

The present data showed that transcriptional activity of AP-1, a MAPK response molecule, was dose-dependently inhibited subsequent to BM treatment. Simultaneously, the p-JNK and p38 expression was suppressed, whereas a limited effect was exhibited on the phosphorylation of ERK1/2 following BM treatment. These results indicated that the NF- κ B and MAPKs pathways were involved in the anti-inflammatory activities of BM.

A critical activity of macrophages in the inflammation process are their ability to migrate in response to stimuli. The interaction between macrophages and chemoattractants not only starts a rapid and directed movement, but is also associated with a complicated range of cellular events, including ion flux changes, integrin avidity alterations, superoxide production and lysosomal enzyme secretion (42). Chemokines are an important group of chemoattractants that can be classified into four categories based on their cysteine motifs, namely CXC, CC, CX3C and C. In the present study, a cytokine array was utilized to screen the changes of the chemokines in response to BM administration. The results showed that four cytokines, CCL-2, CCL-3, CCL-5 and sTNFR, exhibited significant reductions in expression after BM treatment, and that CCL-5 had the highest inhibition ratio. Mounting evidence records the abilities of CCL-2, 3 and 5 in recruiting macrophages into inflammation sites (43-45), and the present data also revealed that the suppressive effects of BM on NF- κ B activity were relieved following the addition of CCL-5, indicating that CCL-5 may be the main molecular target in the anti-inflammatory activity of BM. Previous studies demonstrated that the IKK inhibitor repressed the mRNA levels of CCL-5 by ~81%, and these results indicated that CCL-5 may also act as an upstream factor enhancing NF- κ B activity (46). The present study also validated the ability of BM to inhibit the CCL-5 expression and NF- κ B activity in GLM samples; the results not only confirmed the anti-inflammatory effects of BM *ex vivo*, but also suggested that CCL-5 overexpression may contribute to the pathogenesis of GLM, and that the underlying molecular mechanism is closely associated with the activation of the NF- κ B pathway.

Among the four identified compounds in BM extracts, chlorogenic acid and berberine are the compounds most reported to exhibit anti-inflammatory activities. Chlorogenic acid has been validated to be effective in inhibiting multiple inflammation models, including atopic dermatitis, liver and kidney injury, gout and rheumatoid arthritis (47-50). The expression levels of IL-1 β , IL-6, TNF- α and iNOS were downregulated after chlorogenic acid administration. Meanwhile, chlorogenic acid could also restore the expression of superoxide dismutase, catalase and malondialdehyde in the injured liver tissue, whereas it inhibited the transcription of NF- κ B signaling and keratinocyte chemoattractants (49,51). Berberine was also recorded to exhibit significant anti-inflammatory activities, including proinflammatory cytokine suppression, NF- κ B/MAPK signaling blockade, oxidative stress reduction and apoptotic induction (52-56). Therefore, chlorogenic acid and berberine may synergistically contribute to the anti-inflammatory activities of BM. However, since one herb may contain hundreds of bioactive compounds, it remains necessary to investigate the bioactive compounds in BM using bioactivity-guided fractionation and high-throughput technologies.

Taken together, the results of the present study firstly validated the anti-inflammatory activities and mechanisms of BM for GLM treatment. Meanwhile, novel light was shed on the potential link between CCL-5 and GLM pathogenesis. However, further research is required to investigate the bioactive compounds in BM by targeting CCL-5, and to investigate the clinical significance of CCL-5 in GLM treatment and model establishment.

Acknowledgements

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