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Dihydroberberine, a hydrogenated derivative of berberine firstly identified in Phellodendri Chinese Cortex, exerts anti-inflammatory effect via dual modulation of NF-κB and MAPK signaling pathways

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ABSTRACT

Dihydroberberine (DHB), a hydrogenated derivative of berberine (BBR), has been firstly identified in Phellodendri Chinese Cortex (PC) by HPLC-ESI-MS/MS. Nowadays most researches on PC focus on its main components like BBR, however, the role of its naturally-occurring derivatives remains poorly defined heretofore. The present work aimed to comparatively evaluate the in vivo anti-inflammatory properties and mechanisms of DHB and BBR in three typical inflammatory murine models. The results showed that DHB effectively mitigated acetic acid-induced vascular permeability, xylene-elicited ear edema and carrageenan-caused paw edema. Meanwhile, DHB markedly attenuated the inflammatory cell infiltration in pathological sections of ears and paws. DHB was also observed to significantly decrease the production and mRNA expression levels of IL-6, IL-1β, TNF-α, NO (iNOS) and PGE2 (COX-2), increase the release of IL-10, and inhibit the activation of NF-κB and MAPK signaling pathways. The anti-inflammatory effect of DHB was weaker than that of BBR. The results might further contribute to unraveling the pharmacodynamic basis of PC and support its ethnomedical use in the treatment of inflammatory diseases. DHB possesses good potential to be further developed into a promising anti-inflammatory alternative, and can serve as a lead template for novel anti-inflammatory candidate.

1. Introduction

Inflammation is a physiological response of immune defense system to protect the body from noxious stimuli such as infections, allergens and trauma [1,2]. However, exaggerated inflammatory response not only leads to the release of inflammation-related mediators and the activation of NF-κB and MAPK signaling pathways [3], but also results in diabetes, cardiovascular diseases, rheumatoid arthritis, cancer and even death [4,5]. Nowadays, despite nonsteroidal anti-inflammatory drugs are frequently used to treat inflammatory diseases, their clinical applications are often restricted due to their side effects [2]. Identification of promising anti-inflammatory alternatives derived from medicinal herbal plants is becoming a vital area of research for the development of novel anti-inflammatory agents.

Phellodendri Chinese Cortex (PC), the dried bark of *Phellodendron chinense* Schneid., is a famous traditional Chinese materia medica extensively used for the treatment of varied inflammatory diseases such as gastroenteritis, gastric ulcer, and rheumatoid arthritis, etc. [6]. Modern study has suggested that PC possesses appreciable anti-inflammatory effect [6], and the protoberberine alkaloids contained therein are

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regarded as its major active components and are of vast medical interest. Berberine (BBR), the most common protoberberine alkaloid and major active constituent of PC, has been found to display multiple biological activities including anti-inflammatory [7], anti-diabetic [8], and anti-cancer effects [9]. However, current biological studies on protoberberine alkaloids have predominantly concentrated on its major constituent BBR, while limited attempt has been initiated to explore the biological activities of its natural derivatives like dihydroberberine (DHB). Indeed, pharmacokinetic analyses have indicated that nitroreductases of the gut microbiota reduces BBR to its absorbable form DHB, which displays improved absorption and enhanced oral bioavailability [10]. In addition, the in vivo bioactivities of DHB have been reported to be stronger than those of BBR, such as anti-atherosclerosis [11], anti-adiposity, and anti-diabetes [12], etc.

In a continuing effort to more fully characterize the anti-inflammatory potential of natural candidates, in the present work, we identified DHB, a natural reduced derivative and gut-bacteria metabolite of BBR, in ethyl acetate extract of PC by HPLC-ESI-MS/MS, comparatively scrutinized their anti-inflammatory effects and delineated the molecular mechanisms of action. To the best of our knowledge, this is the first identification of dihydroprotoberberine in PC and the pioneering endeavor comparatively exploring the anti-inflammatory properties of protoberberine alkaloid and its hydrogenated derivative in typical acute inflammation murine models.

2. Materials and methods

2.1. Plant material

Phellodendri Chinese Cortex samples, obtained from Lingnan Chinese Herbal Medicine Co., Ltd. (Guangzhou, China), were authenticated by Prof. Zi-Ren Su of Guangzhou University of Chinese Medicine (Guangzhou, China). A voucher specimen (No. 20170901) was deposited in the herbarium of Mathematical Engineering Academy of Chinese Medicine, Guangzhou University of Chinese Medicine (Guangzhou, China).

2.2. Chemicals and reagents

Standards of berberine (BBR, $C_{20}H_{18}NO_4$) and dihydroberberine (DHB, $C_{20}H_{19}NO_4$) were provided by Chengdu Purechem-Standard Co., Ltd. (Sichuan, China). Indomethacin, Evans blue and carrageenan were obtained from Sigma Aldrich (Saint Louis, USA). Antibodies against beta-actin (β-actin), Histone H3 (H3), IkappaB-alpha (IκBα), phospho-IkappaB alpha (p-IκBα), p65 (p65), p38 mitogen-activated protein kinases (p38), phospho-p38 mitogen-activated protein kinases (p-p38), extracellular signal-regulated kinase (ERK), phospho-extracellular signal-regulated kinase (p-ERK), c-jun N-terminal kinase (JNK), phospho-c-jun N-terminal kinase (p-JNK), and horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG) were purchased from Affinity Biosciences (Cincinnati, USA). Test kits for IL-1β, IL-6, IL-10, TNF-α, and prostaglandin E_2 (PGE_2) levels were provided by Cusabio (Wuhan, China). All reagents unless specified were of analytic grade.

2.3. HPLC-ESI-MS/MS analysis of Phellodendri Chinese Cortex

In order to explore the components of PC, the ethyl acetate extract of PC were prepared with the ultrasonic technique and analyzed by HPLC-ESI-MS/MS. In this test, chromatographic analyses were implemented on the Agilent UPLC system (Agilent Technologies, Palo Alto, CA, USA). The Phenomenex Gemini C_{18} column (100 mm × 2.0 mm, 3 μm) was used for the chromatographic separation. The mobile phase consisted of solvent A (acetonitrile) and B (0.1% formic acid aqueous solution with 10 mM ammonium). The gradient elution conditions were as follows: 5–10% A from 0 to 5 min, 10–40% A from 5 to 15 min, 40–60% A from 15 to 20 min, and 60–90% A from 20 to 25 min. The mobile phase flow rate was 0.4 mL/min and the injection volume was 2 μL [13]. The ESI-MS spectrometry was conducted with a Multiquant™ Software system and a Triple TOF5600 system (AB SCIEX, Framingham, MA, USA) equipped with an electrospray ionization source. The conditions of mass spectrometry were as follows: positive ion mode ($[M + H]^+ = 338.1$); spray voltage, −4500 kV; ion source temperature, 500 °C; curtain gas, 35 psi; ion source gas, 55 psi; scanned range, 40 to 1000 mz; collision energy, −40 eV, collision cell exit voltage, −100 eV. The mass spectra plot was recorded in automatic mode.

Table 1
Sequences of primers used in RT-PCR assay.

Target gene		Primer sequences (5′-3′)
iNOS	Forward	ATCTTGGAGCGAGTTGTGGATTGTC
	Reverse	GGTTGTTGCTGAACTTCCAGTCATTG
COX-2	Forward	CTGGTGCCTGGTCTGATGATGTATG
	Reverse	AGCTGTACTCCTGGTCTTCAATGTTG
TNF-α	Forward	CACCACGCTCTTCTGTCTACTGAAC
	Reverse	CATCGGCTGGCACCACTAGTTG
IL-1β	Forward	TTCAGGCAGGCAGTATCACTCATTG
	Reverse	TGTCGTTGCTTGGTTCTCCTTGTAC
IL-6	Forward	AGACTTCCATCCAGTTGCCTTCTTG
	Reverse	AGTTGTTCTTCATGTACTCCAGGTAGC
IL-10	Forward	ACATACTGCTAACCGACTCCTTAATGC
	Reverse	CTTCACCTGCTCCACTGCCTTG
β-Actin	Forward	ATCTGGCACCACACCTTCTACAATG
	Reverse	CACGCTCGGTCAGGATCTTCATG

2.4. Extraction and isolation of dihydroberberine

In the present work, DHB was isolated from Phellodendri Chinese Cortex based on previous investigation and our prior trial [14,15]. Briefly, the dried powder of Phellodendri Chinese Cortex (2 kg) was macerated in ethyl acetate (4 L) and extracted with the ultrasonic technique. After filtration and evaporation, the residue was chromatographed on the silica gel column using methanol-chloroform mixed liquor with increasing polarity, and the methanol fraction was purified on Sephadex LH-20 column with methanol-chloroform-water mixed liquor. Identical fraction was further purified with preparative thin layer chromatography to collect yellow DHB crystals. The chemical structure of DHB was elucidated by comparing its spectral data (MS and NMR) with previous reports [13,16].

2.5. Synthesis of dihydroberberine

Due to the limited content of dihydroberberine in PC, DHB was synthesized and identified for further bioactivity evaluation. In the present work, DHB was synthesized according to previous method with some modifications [16]. Briefly, 5% sodium hydroxide solution (27 mL) containing sodium borohydride (2.7 g) was added to a stirred solution of berberine chloride (11.06 g) and potash (13.1 g) in methanol (150 mL) dropwise. After the reaction mixture was stirred at ambient temperature for 3 h, the precipitation was filtered and scrubbed with 30% ethanol (200 mL) followed by 80% ethanol (100 mL). The green–brown crystals were filtered and dried at 30 °C, and were then collected to afford 9.4 g (yield: 85%) DHB. The spectral data (MS and NMR) were in agreement with those of the standard of dihydroberberine and previous studies [13,16]. The purity was determined to be > 98% by HPLC.

2.6. Experimental animals and groupings

Kunming (KM) mice of either sex (18–22 g) were obtained from Animal Experimental Center of Guangzhou University of Chinese

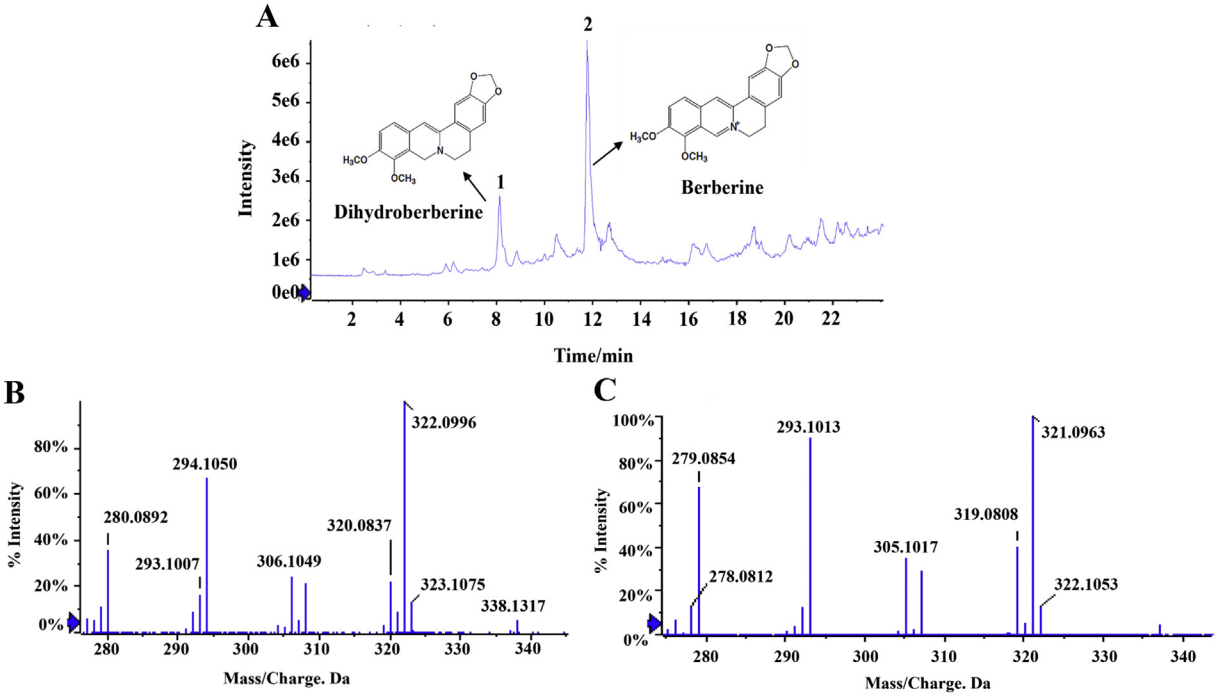

Fig. 1. Total ion chromatogram of Phellodendri Chinese Cortex ethyl acetate extract (A) and MS^2 mass spectra of dihydroberberine (B) and berberine (C) in the positive ion mode.

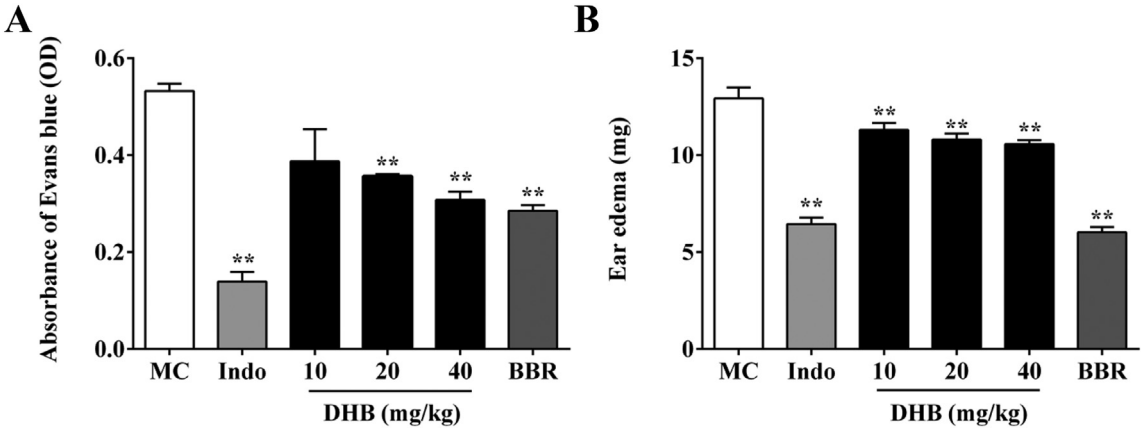

Fig. 2. Effect of DHB on acetic acid-induced abdominal capillary permeability (A) and xylene-induced ear edema (B) in mice. MC: model control group; Indo: 10 mg/kg indomethacin; BBR: 20 mg/kg berberine; DHB: 10, 20 and 40 mg/kg dihydroberberine. Values given are mean ± S.E.M. ($n = 10$). *$p < 0.05$ and **$p < 0.01$ vs. MC group.

Medicine (No. 44007200042620). Animals were housed under specific-pathogen-free conditions of 22 ± 2 °C and 55 ± 2% humidity with a 12-h light/dark cycle, and fed with forage and clean water ad libitum. All animal experimental protocols were approved by the Animal Experimental Ethics Committee of Guangzhou University of Chinese Medicine (No. dspf 2017098) and conducted in accordance with the National Institutes of Health (NIH) guide for the Care and Use of Laboratory Animals.

After one-week acclimatization, male and female mice were randomly divided into 7 groups: normal control group (NC), model control group (MC), indomethacin-treated group (Indo, 10 mg/kg), berberine-treated group (BBR, 20 mg/kg), and dihydroberberine-treated groups (DHB, 10, 20 and 40 mg/kg), respectively. All drugs were dissolved in 0.5% Tween 80 and administered intragastrically once daily for 7 consecutive days. The dosages of agents used in this experiment were adopted according to our prior trial and previous research [17].

2.7. Acetic acid-induced vascular permeability in mice

Acetic acid-caused abdominal capillary permeability model, one of typical acute inflammatory models, was established according to previous study [18]. Briefly, 60 min after the last administration, mice (10 mice each group) except those in the NC group were intravenously injected with 1% Evans blue in physiological saline solution, followed by an intraperitoneal injection of 0.6% acetic acid at 0.1 mL/10 g body weight. Twenty minutes later, mice were sacrificed and the Evans blue infiltrated into the enterocoelia was washed several times with 5 mL normal saline solution. The leaner of enterocoelia was centrifuged at 3000 r/min for 15 min, and was determined at 590 nm to calculate the inhibitory rate of the permeability increment.

2.8. Xylene-caused edema in mice ears

The xylene-induced acute inflammatory model was established in

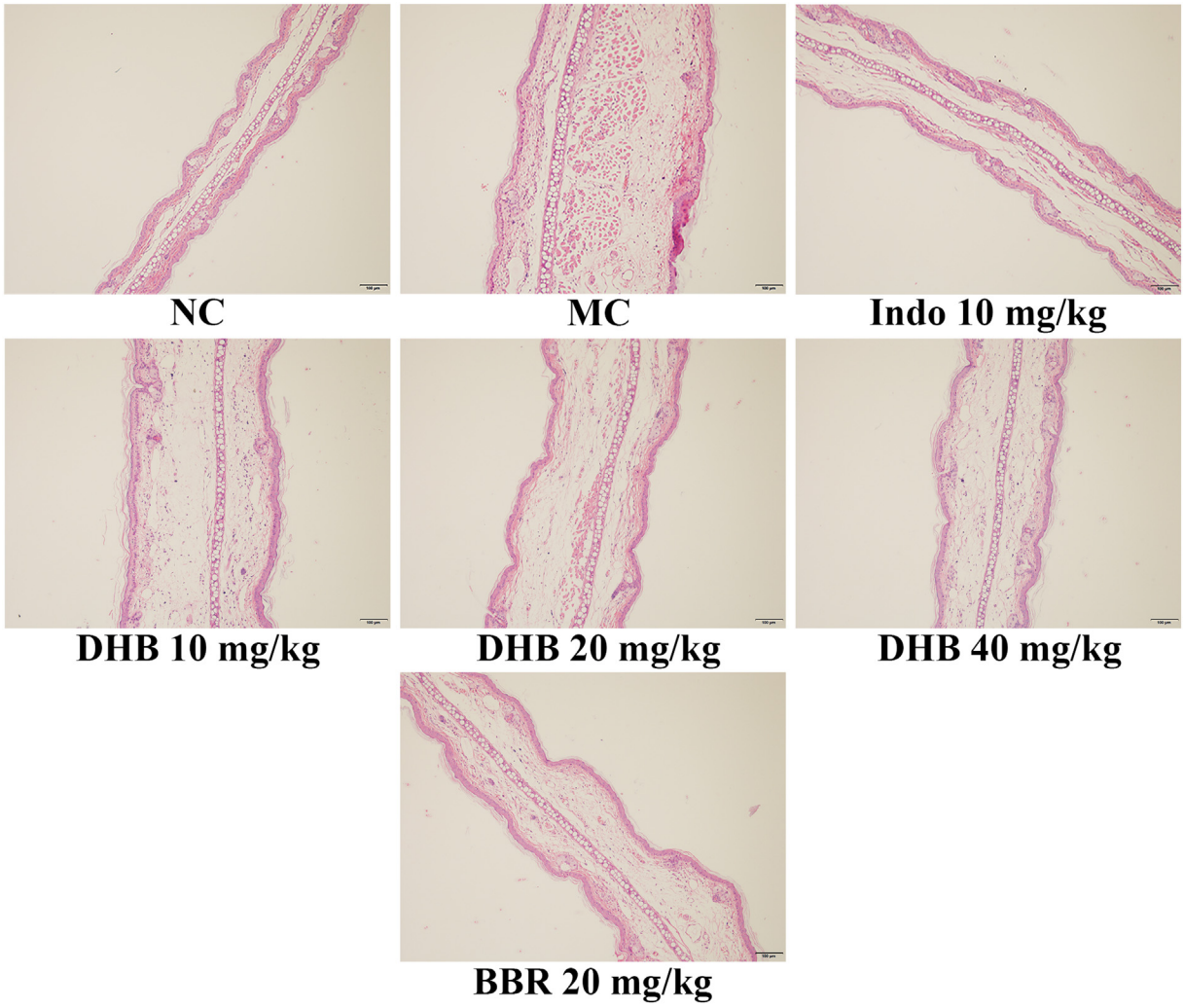

Fig. 3. Representative histological sections of xylene-treated ears in mice (magnification × 200). NC: normal control group; MC: model control group; Indo: 10 mg/kg indomethacin; BBR: 20 mg/kg berberine; DHB: 10, 20 and 40 mg/kg dihydroberberine. The demonstrated sections are typical representatives of three animals each group.

this study following the method described previously [19]. In brief, 1 h after the final treatment, the right ears of all mice were treated with 20 μL xylene, while the left ears were devoid of any treatment. One hour later, animals were sacrificed and 2 ear biopsies were punched by biopsy punch with a diameter of 8 mm for sample collection and weight measurement. The weight difference between 2 ears from the same animal was used to measure the effect of DHB on xylene-caused ear edema. After measurement of the ear weight, samples were immersed in 4% paraformaldehyde for tissue fixation. Tissue sections were cut into 5 μm, embedded in paraffin, and stained with hematoxylin and eosin following routine procedure to evaluate the severity of inflammation.

2.9. Carrageenan-caused edema in mice paws

Another well-established acute inflammatory model induced by carrageenan in mice paws was adopted following reported regime with minor modifications [20]. One hour after the last administration, subcutaneous tissue of right hind paw in mice was injected with 1% carrageenan solution except mice in the normal control group. To determine the effect of DHB on paw edema induced by carrageenan, the volume of the mouse right hind paw in each group was determined with MK101CMP plethysmometer before and after the carrageenan injection at different time points (0, 1, 2, 3, 4, 5 and 6 h). The edema rate and suppression ratio of paw edema were measured following the routine manner.

In the following experiment, the rest mice were subjected to the same experimental protocol abovementioned. Four hours after the carrageenan injection, mice were euthanized, and the right hind paws were collected for further mechanism investigation.

2.9.1. Histopathological examination

For standard histopathological inspection, the paw biopsies obtained from the mice were soaked in 4% paraformaldehyde immediately. One week later, hematoxylin and eosin were applied to stain paw tissues embedded in paraffin. The pathological biopsies were observed under Olympus BX53 microscope (Olympus, Tokyo, Japan).

2.9.2. Measurement of inflammatory mediators production

To measure the levels of inflammation-related mediators in the paw, specimens from the right hind paw were homogenized and centrifuged according to the manufacturer's instructions. The supernatant was collected and frozen at −80 °C until use. The levels of IL-6, IL-10, IL-1β, TNF-α and PGE_2 were measured according to the protocols of corresponding enzyme linked immunosorbent assay (ELISA) kits. In addition, the content of nitrite was measured using the Griess reagent to analyze the nitric oxide (NO) level.

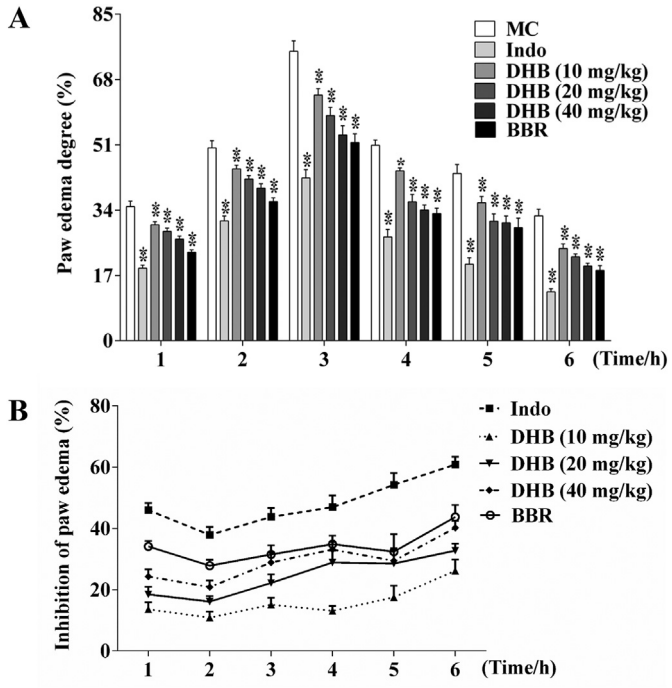

Fig. 4. Effect of DHB on carrageenan-induced paw edema in mice. MC: model control group; Indo: 10 mg/kg indomethacin; BBR: 20 mg/kg berberine; DHB: 10, 20, and 40 mg/kg dihydroberberine. Paw edema degree (A) and inhibition rate of paw edema (B) were evaluated after carrageenan treatment as compared with the MC group. Values given are mean ± S.E.M. ($n = 8$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. MC group.

2.9.3. Real-time polymerase chain reaction analysis

Real-time polymerase chain reaction (RT-PCR) was carried out to quantitate the mRNA expression levels of IL-6, IL-10, IL-1β, TNF-α, cyclooxygenase-2 (COX-2), and inducible type nitric oxide synthase (iNOS). TRIzol reagent (Invitrogen, USA) was used to harvest the total RNA from paw samples according to the manufacturer's protocol. The purified RNA was transformed into cDNA according to the instruction of the Hiscript II QRT SuperMix for RT-PCR. The RT-PCR was performed using the ChamQ SYBR qPCR Master Mix with a Real-Time PCR Detection System of Bio-rad CFX96 Touch and the amplification condition was employed as mentioned previously [21]. The specific primer sequences used in this assay were shown in Table 1. The mRNA expression level was computed with the $2^{-\triangle\triangle CT}$ method and normalized to β-actin.

2.9.4. Western blot analysis

Western blot analysis was carried out to evaluate the effect of DHB on NF-κB and MAPK signaling pathways. The total protein was extracted from the paw specimens with RIPA lysis buffer supplemented with protease inhibitor cocktail while the nuclear and cytoplasmic proteins were prepared according to the instruction of Nuclear and Cytosol Protein Extraction Kit (KeyGEN BioTECH, Nanjing, China). The protein concentrations were measured with the BCA protein assay kit. For Western blot, equal amounts of proteins were separated with appropriate concentrations of SDS-PAGE gel and transferred to PVDF membrane. Subsequently, the membrane was blocked with bovine serum albumin or skim milk powder, followed by incubation with primary antibodies at 4 °C overnight. On the next day, horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse IgG or anti-rabbit IgG) was used to incubate the membrane at room temperature for 2 h. Finally, the protein bands were observed with enhanced chemiluminescence detection reagents (Bio-Rad, USA). The expression level of each protein band was analyzed with the Quantity One Software of Bio-Rad.

2.10. Statistical analysis

The data were shown as mean ± standard error of the mean (S.E.M.) and analyzed with SPSS 23.0 (SPSS Inc., USA). Differences among groups were analyzed by one-way analysis of variance followed by LSD test or Duncan test. Differences were considered to be statistically significant at $p < 0.05$. Furthermore, Student's *t*-test was used to evaluate the difference between two groups.

3. Results

3.1. Composition analysis of Phellodendri Chinese Cortex

The HPLC-ESI-MS/MS chromatograms of PC ethyl acetate extract were shown in Fig. 1. The total ion chromatogram showed that peaks 1 and 2 were predominantly observed in the ethyl acetate extract of PC (Fig. 1A). According to previous reports and corresponding standard compound, peaks 1 and 2 were identified as dihydroberberine (retention time, 8.12 min; [M] + ion, *m/z* 338.1; the characteristic fragment ions, *m/z* 322.0996, *m/z* 306.1049, *m/z* 294.1050 and *m/z* 280.0892) [13] and berberine (retention time, 11.77 min; [M] + ion, *m/z* 337.1; the characteristic fragment ions, *m/z* 321.0963, *m/z* 305.1017, *m/z* 293.1013 and *m/z* 279.0854) [22], respectively. The content of DHB and BBR was 22.4 and 43.8 μg/g, respectively (Fig. 1B and C).

3.2. Effect of DHB on acetic acid-caused vascular permeability

As illustrated in Fig. 2A, pretreatment with DHB (20 and 40 mg/kg) resulted in a remarkable reduction in acetic acid-induced dye leakage as compared with the MC group (inhibition rate of 33.1% and 42.3%, respectively, $p < 0.01$). While 10 mg/kg DHB did not show significant inhibitory effect ($p > 0.05$). Pretreatment with Indo and BBR exerted more potent suppressive effect against the extravasation of Evans blue with the suppression ratio of 73.9% and 46.5% ($p < 0.01$), respectively.

3.3. Effect of DHB on xylene-caused mouse ear edema

As shown in Fig. 2B, obvious ear edema induced by xylene was observed in the MC group as anticipated. Nevertheless, as compared with the MC group, pretreatment with 10, 20 and 40 mg/kg DHB exerted significant suppressive effect on xylene-caused ear edema (32.3%, 33.9% and 40.3%, respectively, all $p < 0.01$). While Indo (10 mg/kg) and BBR (20 mg/kg), serving as the positive controls, significantly decreased the swelling with the inhibition rate of 54.22% and 56.44% (both $p < 0.01$), respectively. The histopathologic analysis was shown in Fig. 3. Result indicated that the ear biopsy specimens exposed to xylene showed significant connective tissue loosening, inflammatory cell infiltration and increment of dermis thickness. However, when compared with the MC group, pretreatment with 3 tested doses of DHB exerted distinct improvement in dermis thickness and inflammatory cell infiltration. Pretreatment with Indo and BBR also significantly ameliorated the inflammatory symptoms abovementioned.

3.4. Effect of DHB on carrageenan-caused mouse paw edema

After subcutaneous carrageenan injection into the right hind paw of mice, the paw volume increased remarkably (up to 3 h) in a time-dependent manner (Fig. 4A). However, when compared to the MC group, all phases of paw edema induced by carrageenan were markedly inhibited by pretreatment with DHB, BBR and Indo (Fig. 4A and B). The suppression ratio of the DHB group (10, 20 and 40 mg/kg) increased to 26.2%, 32.8%, and 40.2% at 6 h after carrageenan injection (all $p < 0.01$), respectively. While pretreatment with Indo (10 mg/kg) and BBR (20 mg/kg) resulted in 60.9% and 43.7% inhibitory rate against carrageenan-induced paw edema ($p < 0.01$), respectively (Fig. 4B).

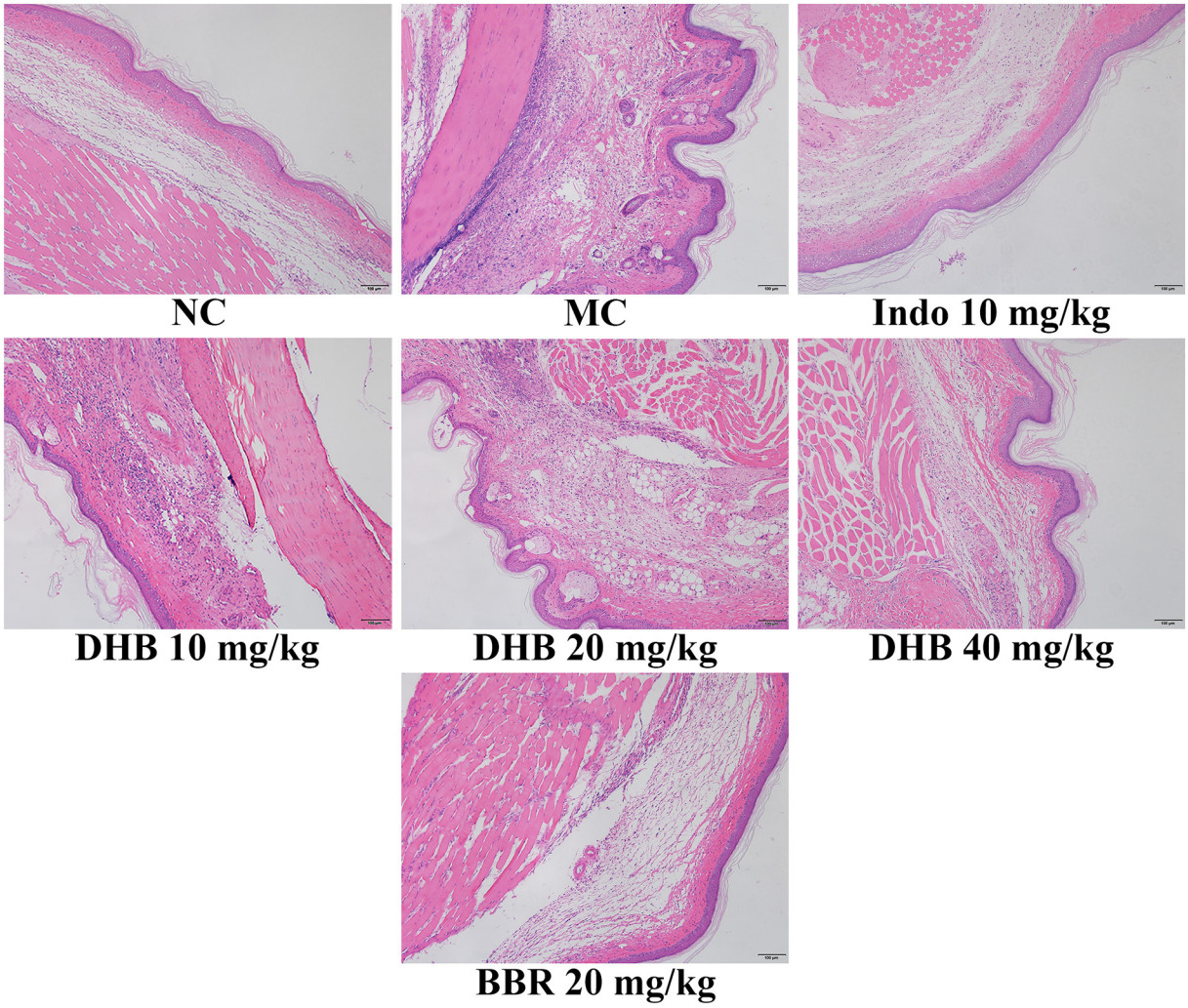

Fig. 5. Histological sections of carrageenan-treated paws in mice (magnification ×200). NC: normal control group; MC: model control group; Indo: 10 mg/kg indomethacin; BBR: 20 mg/kg berberine; DHB: 10, 20, and 40 mg/kg dihydroberberine. The demonstrated sections are typical representatives of three animals each group.

3.4.1. Histopathologic analysis of paw tissue

As revealed in Fig. 5, as compared to the NC group, the paw specimens of the MC group exhibited remarkable inflammatory response, such as dropsy, tissue destruction, and inflammatory cell infiltration. However, pretreatment with DHB (10, 20, and 40 mg/kg) significantly alleviated the swelling degree, tissue damage extent and inflammatory infiltration in carrageenan-treated mice. BBR (20 mg/kg) and Indo (10 mg/kg) showed more profound effect in alleviating the inflammatory symptoms as compared to DHB.

3.4.2. Effect of DHB on the levels of inflammatory mediators

As illustrated in Fig. 6, carrageenan treatment dramatically increased the production of NO (Fig. 6A), PGE_2 (Fig. 6B), TNF-α (Fig. 6C), IL-1β (Fig. 6D) and IL-6 (Fig. 6E) (all $p < 0.01$) in parallel to the NC group. By contrast, the levels of these mediators were obviously decreased ($p < 0.05$ or $p < 0.01$) by pretreatment with DHB (20, 40 mg/kg), BBR (20 mg/kg) and Indo (10 mg/kg), when compared with those of the MC group. Albeit 10 mg/kg DHB significantly suppressed the release of NO ($p < 0.01$), no obvious effects on the production of IL-6, IL-1β, TNF-α and PGE_2 were observed. The level of IL-10 in the MC group was significantly decreased ($p < 0.01$) as compared with that of the NC group (Fig. 6F). However, pretreatment with DHB (10, 20 and 40 mg/kg), BBR, and Indo all remarkably increased the release of IL-10 ($p < 0.05$ or $p < 0.01$) as compared to the MC group.

3.4.3. Effect of DHB on the mRNA expression of inflammatory mediators

As shown in Fig. 7, when compared with the NC group, the gene expression of iNOS (Fig. 7A), COX-2 (Fig. 7B), TNF-α (Fig. 7C), IL-1β (Fig. 7D), and IL-6 (Fig. 7E) (all $p < 0.01$) was significantly up-regulated after injection of carrageenan. When compared with the MC group, pretreatment with DHB (20 and 40 mg/kg), BBR, and Indo considerably suppressed ($p < 0.05$ or $p < 0.01$) the mRNA expression of these mediators. While DHB at 10 mg/kg only exerted conspicuous reduction on the expression of IL-1β ($p < 0.05$), COX-2, and iNOS ($p < 0.01$). There was a remarkable decline of the IL-10 gene expression ($p < 0.01$) in carrageenan-treated group in contrast with the NC group. Whereas DHB (10, 20 and 40 mg/kg) and positive drugs (10 mg/kg Indo and 20 mg/kg BBR) resulted in prominent augmentation on the expression of IL-10 when compared with the MC group (all $p < 0.01$) (Fig. 7F).

3.4.4. Effect of DHB on the NF-κB and MAPK signaling pathways

Given that NF-κB and MAPK are the major transcriptional regulators for inflammatory mediators [23,24], the effects of DHB on activation of NF-κB and MAPK pathways were detected. As shown in Fig. 8A, carrageenan treatment induced a significant increase in nuclear translocation of p65, while pretreatment with Indo (10 mg/kg), BBR (20 mg/kg) and DHB (10, 20 and 40 mg/kg) significantly suppressed p65 translocation from cytoplasm to nucleus as compared to the MC group.

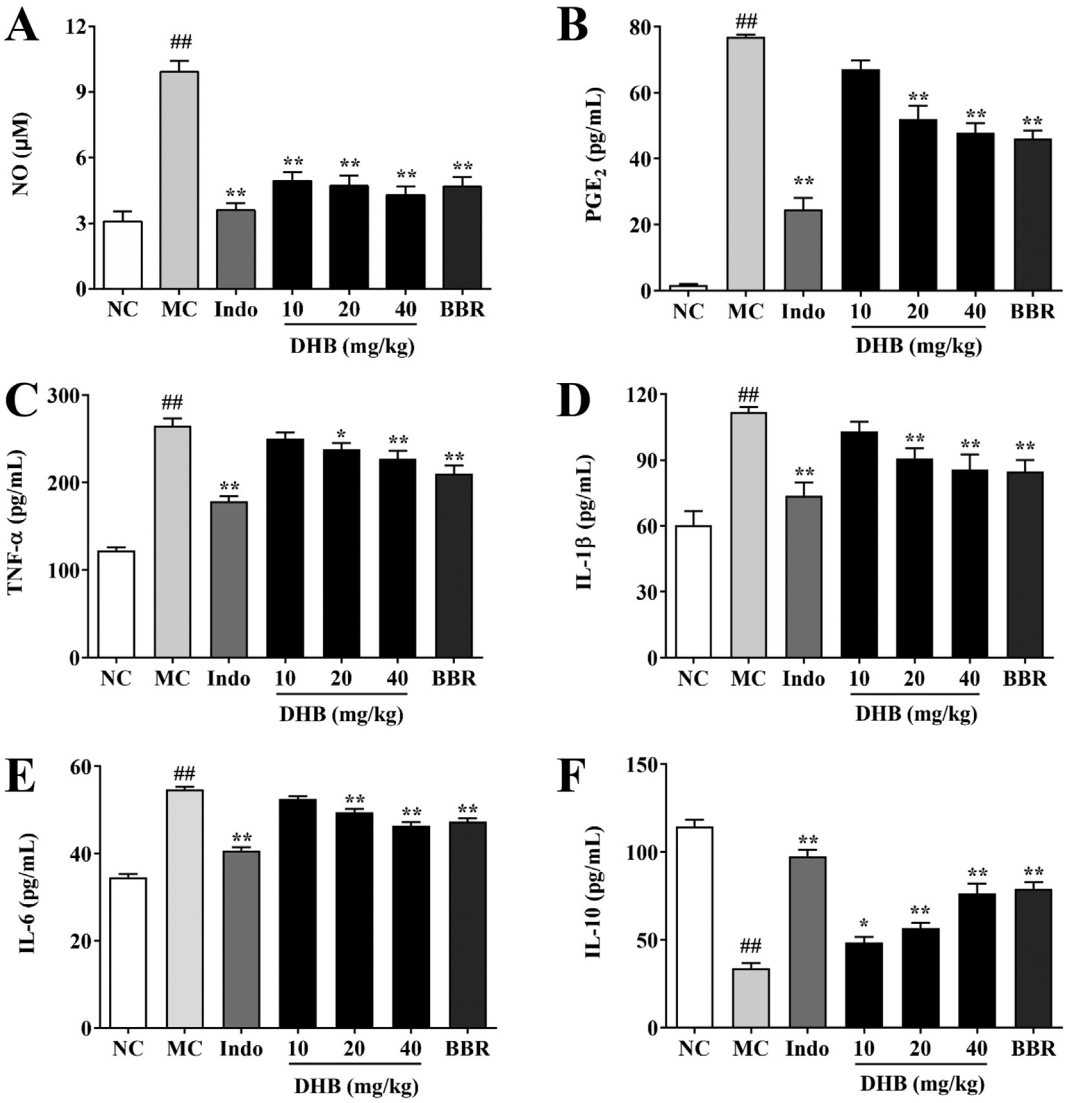

Fig. 6. Effect of DHB on the levels of NO (A), PGE_2 (B), TNF-α (C), IL-1β (D), IL-6 (E) and IL-10 (F) in carrageenan-treated mice paws. NC: normal control group; MC: model control group; Indo: 10 mg/kg indomethacin; BBR: 20 mg/kg berberine; DHB: 10, 20, and 40 mg/kg dihydroberberine. Values given are mean ± S.E.M. ($n = 8$). $\#\#p < 0.01$ vs. NC group. $*p < 0.05$ and $**p < 0.01$ vs. MC group.

And carrageenan exposure markedly augmented the phosphorylation and degradation of IκBα as compared to the NC group. By contrast, when compared with the MC group, pretreatment with DHB, BBR, and Indo notably inhibited the degradation and phosphorylation of IκBα.

As shown in Fig. 8B, after exposure to carrageenan for 4 h, the phosphorylation of p38 and JNK was significantly enhanced as compared to the NC group. However, no effect on the phosphorylation of ERK was observed. By contrast, compared to the MC group, pretreatment with DHB, BBR, and Indo observably inhibited the phosphorylation of p38 and JNK.

4. Discussion

In recent years, interest in the bio-properties of DHB has been kindled, and numerous studies have suggested that it possesses more profound anti-inflammatory, anti-atherosclerotic [11], and anti-diabetes effects [12] than quaternary BBR. Besides as a naturally occurring hydrogenated derivative of BBR, DHB is also a gut-bacteria metabolite of BBR in vivo, and displays improved absorption and enhanced oral bioavailability [10,11]. Therefore, DHB is considered a potential candidate agent preferable to BBR for the development of new drug. Hydrogenated protoberberine alkaloids are rarely found in natural herbal medicine in comparison to their quaternary salt counterparts, which are commonly extracted with mineral acid and inorganic base [14]. During these processes, hydrogenated protoberberine alkaloids are likely to be converted to their quaternary salt forms. According to the in vivo biosynthetic pathway of protoberberine alkaloids, hydrogenated protoberberine alkaloids can naturally exist inside plants. In the present work, based on previous investigation and our prior trial, extraction with ethyl acetate led to the identification of DHB from PC for the first time.

In continuation of our work on protoberberine alkaloids [17,25,26], pioneering effort was motivated to comparatively explore the in vivo anti-inflammatory effect of DHB and BBR, and elucidate the potential underlying mechanism. In our study, the anti-inflammatory effects of DHB and BBR were evaluated with three common acute inflammatory murine models, which were typically used to investigate the drug candidates with potential anti-inflammatory effect. The results suggested that pretreatment with DHB exhibited significant suppressive effect on acetic acid-induced vascular permeability, xylene-elicited ear edema and carrageenan-caused paw edema in a dose-dependent manner. BBR exhibited stronger anti-inflammatory effect than DHB at the same dose.

To explore the inhibitory mechanism of DHB on acute

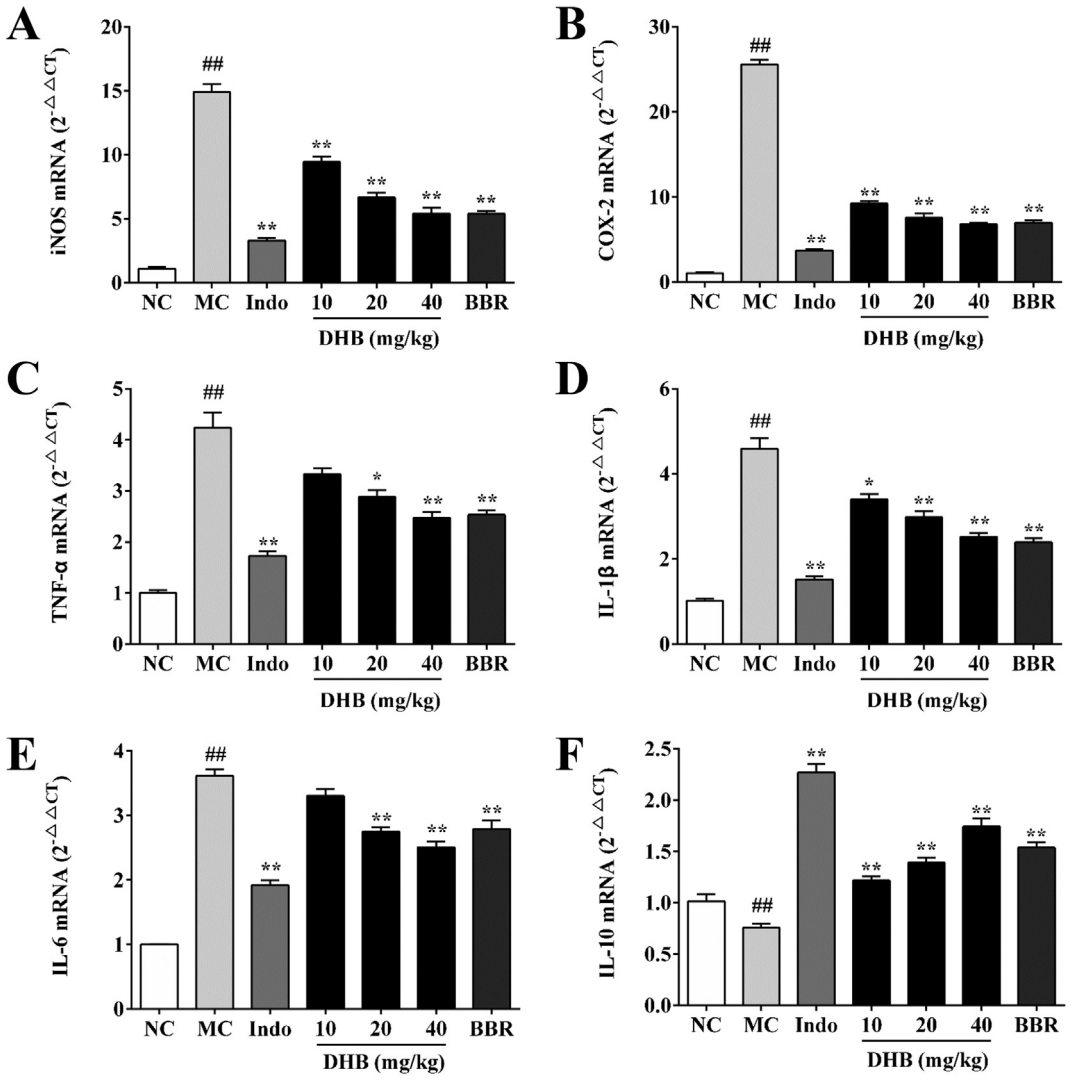

Fig. 7. Effect of DHB on the mRNA expression of iNOS (A), COX-2 (B), TNF-α (C), IL-1β (D), IL-6 (E), and IL-10 (F) in carrageenan-treated mice paws. NC: normal control group; MC: model control group; Indo: 10 mg/kg indomethacin; BBR: 20 mg/kg berberine; DHB: 10, 20 and 40 mg/kg dihydroberberine. Values given are mean ± S.E.M. ($n = 8$). ##$p < 0.01$ vs. NC group. *$p < 0.05$ and **$p < 0.01$ vs. MC group.

inflammation, the carrageenan-induced paw edema mice model was employed. This is a typical and stable acute inflammation model, commonly used to probe the possible mechanism of anti-inflammatory agents. At the early stage of inflammation (0 to 2 h), the progress of paw edema is facilitated primarily by the liberation of histamine, serotonin and bradykinin. While the aggravation of inflammatory reaction is promoted by the expression of cyclooxygenase, prostaglandins and other inflammatory mediators at the late stage (3 to 4 h) [27]. Six hours after carrageenan injection, the inflammatory response could be neglected due to the depletion of kininogen [28]. Therefore, the efficacy and mechanism of DHB on acute inflammation were evaluated within 4 h after carrageenan injection. Our result displayed that paw edema of all phases induced by carrageenan was markedly inhibited by pretreatment with DHB in a dose-dependent manner, and the efficacy of BBR was more pronounced than that of DHB. Consistent with the foregoing result, histopathological analysis proved that pretreatment with DHB significantly abated the carrageenan-induced inflammatory response in mice. The result implied that the anti-inflammatory effect of DHB was potentially associated with the inhibition on the release of cyclooxygenase, prostaglandins and other inflammatory mediators.

Nitric oxide is a crucial signaling molecule produced by the catalytic reaction of inducible type nitric oxide synthase on L-arginine [21]. NO can trigger the elimination of pathogens via production of reactive radicals such as peroxynitrite, and exert regulatory effect on multiply physiological and pathological processes such as inflammation, necrosis and apoptosis [29]. Moreover, researches have shown that NO and iNOS are related to the biosynthesis of COX-2 and directly affect its activity [30]. COX-2 cannot be detected in most tissues in the physiological condition, however, it would be secreted by monocytes, synoviocytes, fibroblasts, megakaryocytes and chondrocytes when stimulated by inflammation and damage [31,32]. In addition, COX-2 participates in the synthesis of prostaglandins, especially PGE_2 [32,33]. PGE_2 is another important inflammatory mediator associated with numerous inflammatory disorders [34]. In the current work, pretreatment with DHB significantly down-regulated the mRNA expression of iNOS and COX-2, and inhibited the release of NO and PGE_2 induced by carrageenan in a dose-dependent manner. DHB supposedly antagonized the NO and PGE_2 production by suppressing iNOS and COX-2 mRNA expression, which presumably contributed to the anti-inflammatory effect of DHB.

IL-6, IL-1β and TNF-α are among the most typical pro-inflammatory mediators, while IL-10 is a well-known anti-inflammatory mediator [35]. TNF-α and IL-1β can activate macrophages, T cells and signaling pathways connected with inflammatory action when the organism is

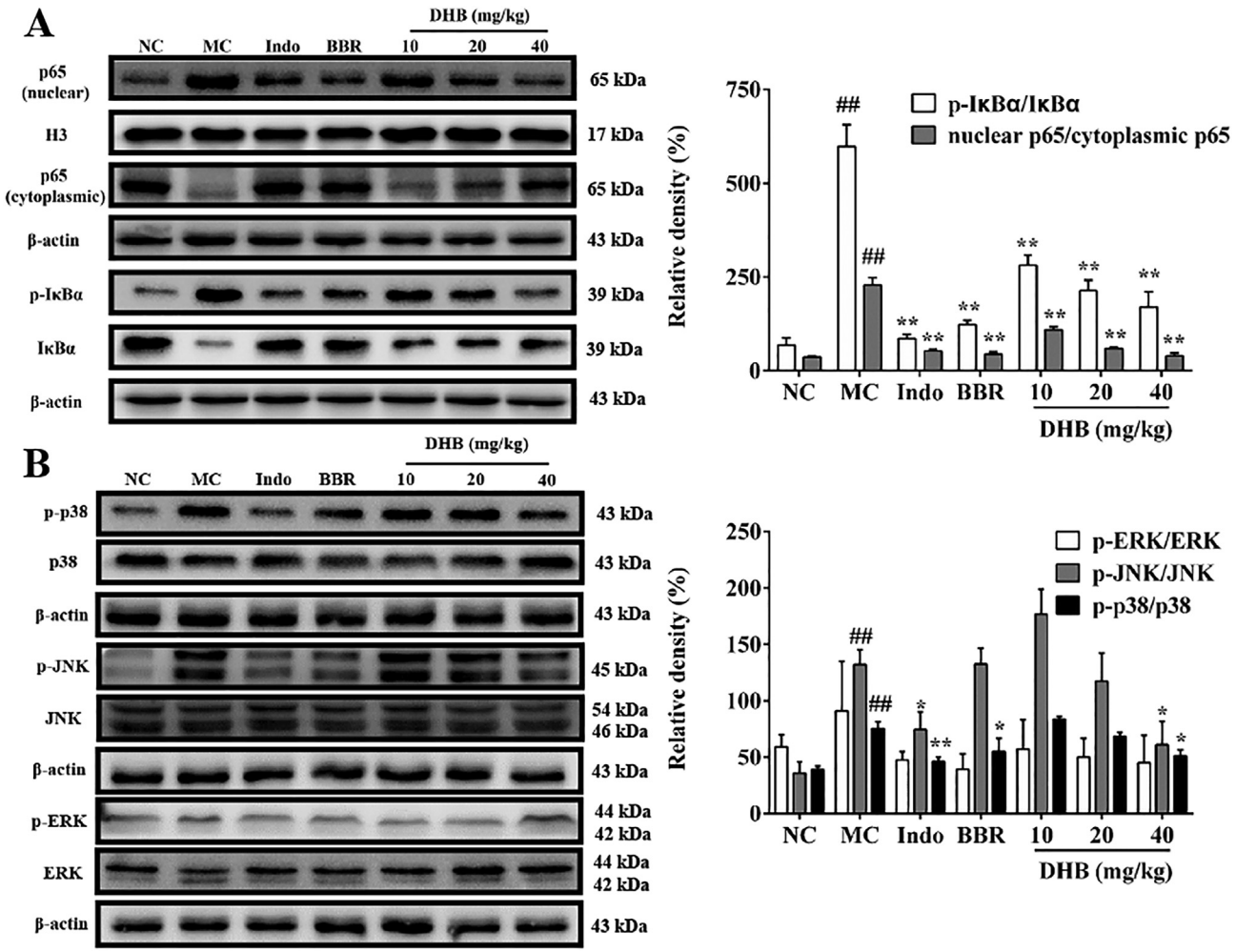

Fig. 8. Effect of DHB on the NF-κB and MAPK signaling pathways in carrageenan-treated mice paws. NC: normal control group; MC: model control group; Indo: 10 mg/kg indomethacin; BBR: 20 mg/kg berberine; DHB: 10, 20 and 40 mg/kg dihydroberberine. (A) The protein expression levels of IκBα, p-IκBα, cytoplasmic p65, and nuclear p65 in the NF-κB signaling pathway. (B) The protein expression levels of p38, p-p38, JNK, p-JNK, ERK, and p-ERK in the MAPK signaling pathway. Values given are mean ± S.E.M. ($n = 3$). ##$p < 0.01$ vs. NC group. *$p < 0.05$ and **$p < 0.01$ vs. MC group.

under attack [35]. To be specific, TNF-α and IL-1β can facilitate the expression of IL-6, iNOS and COX-2 and the generation of PGE_2 [36]. IL-6 is one kind of pro-inflammatory mediators secreted by numerous cell types, including B cells, T cells, mononuclear phagocytes, keratinocytes, endothelial cells, fibroblasts, bone marrow cells and hepatocytes [35]. By augmenting the recruitment of neutrophils and leukocytes, IL-6 conduces to the inflammatory response and multiple disorders [37]. In addition, IL-10, an anti-inflammatory mediator, has been reported to exert inhibitory effect on the release of pro-inflammatory mediators like TNF-α and IFN-γ, and mediates the inflammatory and immune responses [38]. In this study, it was observed that the production and mRNA expression of IL-6, IL-1β and TNF-α were notably suppressed by DHB in a dose-dependent manner, while the level and mRNA expression of IL-10 were remarkably elevated by DHB. Therefore, the anti-inflammatory effect of DHB might be intimately associated with the favorable regulation of anti-inflammatory status.

Previous studies have illustrated that inflammatory mediators including IL-6, IL-1β and TNF-α can boost the activation of MAPK and NF-κB signaling pathways [39]. In turn, the MAPK and NF-κB signaling pathways can also regulate the expression of inflammatory mediators [39]. NF-κB signaling pathway is one of the well-recognized pathways modulating immune and inflammatory responses [23]. Under normal physiological conditions, NF-κB heterodimer consisting of NF-κB1/p50 and Rel A/p65 is normally localized in the cytoplasm via binding to its inhibitor IκB [40]. Once NF-κB is activated, phosphorylation and degradation of IκB will induce the separation of IκB and NF-κB, and the nuclear translocation of NF-κB heterodimer from cytoplasm to nucleus. The transcription of inflammatory mediators, such as IL-6, IL-1β, TNF-α, IL-10, COX-2 and iNOS, will be therefore activated [41]. To determine whether the anti-inflammatory effect of DHB involved the NF-κB signaling pathway, relevant upstream signal molecules of NF-κB were examined. Results revealed that carrageenan treatment activated the NF-κB signaling pathway by promoting the phosphorylation and degradation of IκB and activating the translocation of p65 from cytoplasm to nucleus. Nevertheless, pretreatment with DHB significantly up-regulated the expression of IκBα and p65 (cytoplasmic) and down-regulated the p-IκBα and p65 (nuclear) expression levels. Therefore, DHB might suppress carrageenan-induced inflammation, at least in part, via inhibiting the NF-κB signaling pathway.

Besides the NF-κB signaling pathway, the MAPK pathway consisting of JNK, p38 MAPK and ERK also critically contributes to the development of acute inflammation [42]. ERK, activated by cytokines, phorbol esters, growth factors and other mitogenic stimuli, is related to the modulation of cell meiosis, proliferation and differentiation [42]. The members of JNK family play a key role in inflammation, apoptosis, neural development and various signal transduction [42]. p38 is a vital member of MAPK family to control inflammation [43]. Researches have suggested that p38 can mediate the phosphorylation and activation of

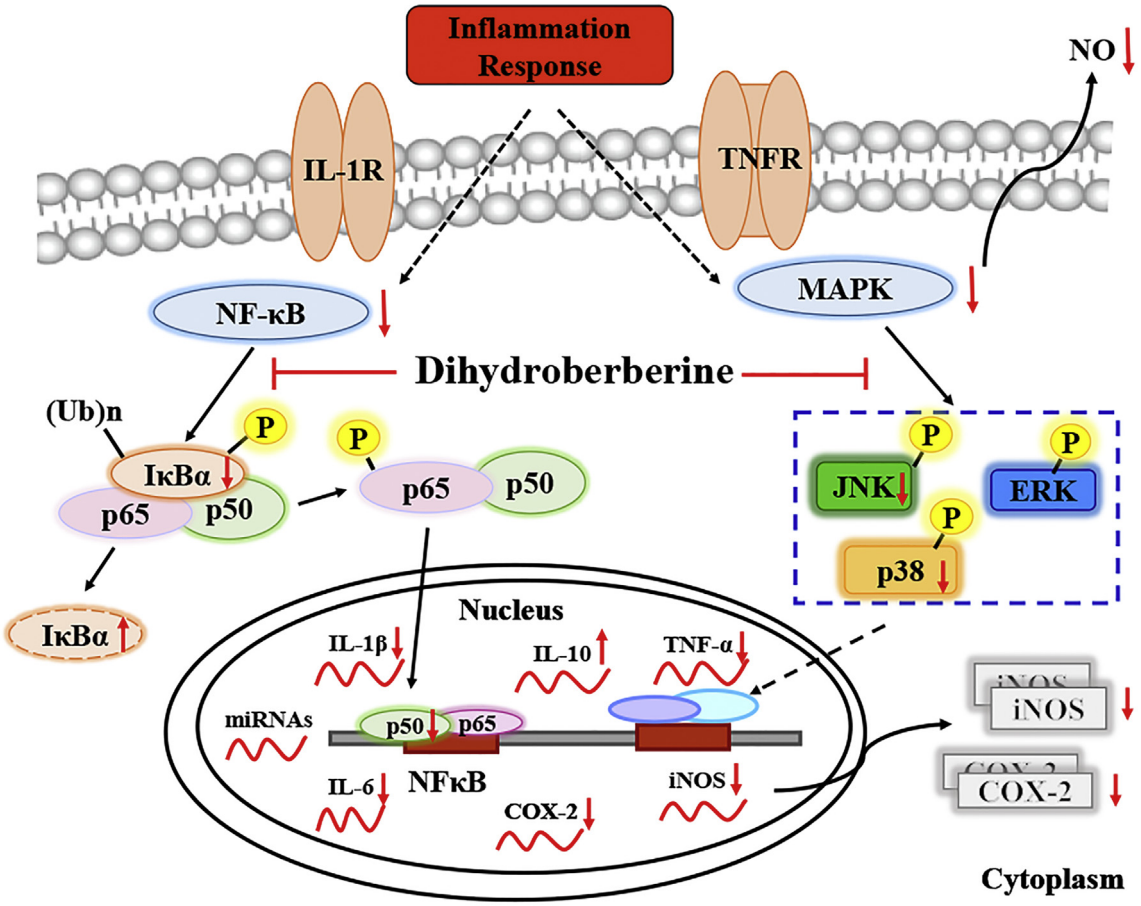

Fig. 9. The proposed anti-inflammatory mechanism of dihydroberberine.

several transcription factors related to inflammation, therefore regulating the production and expression of inflammatory mediators [44]. Our results showed that carrageenan treatment remarkably augmented the phosphorylation of JNK and p38. By contrast, pretreatment with DHB significantly antagonized the phosphorylation of JNK and p38, while exerted no significant effect on ERK. Therefore, the anti-inflammatory effect of DHB might be closely associated with the blockage of NF-κB and MAPK signaling pathways, which was in accord with preceding observations in vitro to some extent [11,42].

Previous researches have reported that the bioactivities of DHB were superior to those of BBR due to enhanced bioavailability [10,11]. Our result indicated that the anti-inflammatory effect of DHB was weaker than that of BBR, which was inconsistent with previous reports suggestive of superior anti-atherosclerosis [11], anti-adiposity, and hypolipidemic [12] activities of DHB over BBR [10–12]. Previous study has revealed that once absorbed, DHB is rapidly converted back to BBR [10], highlighting that this is likely the active moiety. These findings indicate that DHB is in essence an effective vehicle for delivering BBR to the circulation [10], mutually and collectively contributing to the anti-inflammatory effect of PC. Our finding was analogous to the case of curcumin and tetrahydrocurcumin. Research has shown that double bonds play a major role in inactivating NF-κB by curcumin when compared with tetrahydrocurcumin [45].

Structurally speaking, the structure of tertiary amine DHB is derived from quaternary ammonium BBR by reduction of the N7-C8 bond (C═N double bond), and the molecule of DHB lacks classical O—H or N—H donors owing to lack of double bond in the inner quinolizine ring system [16]. Saturation of double bond between N-7 and C-8 (C—N single bond) and the resulting extended conjugated π-electron system of BBR seem to be essential for its anti-inflammatory activity, as could be deduced from the result. Hence, the enhanced anti-inflammatory effect was postulated to be attributed to the presence of aromatization of ring C and quaternary nitrogen (cationic center: the 7-position quaternary ammonium) in BBR, which was congruent with the acetylcholinesterase inhibitory activities of palmatine and tetrahydropalmatine [46]. This observation highlighted the role of quaternary nitrogen contributing to the bioactivities of protoberberine alkaloids, which was in concert with other report to some extent [47].

To the best of our knowledge, this study is the first report that DHB, the hydrogenated derivative of BBR, naturally exists in PC, and also the first identification of hydrogenated protoberberine alkaloids in PC. Our work presents the exception that DHB, the more biologically available derivative of quaternary BBR, exerts weaker anti-inflammatory effect, which enriches our knowledge of comparative bioactive profile of DHB and BBR. However, further in-depth investigation should be merited to provide precise mechanism. And the comparative study on the bioactivity profile of DHB and BBR is warranted in the future work.

5. Conclusion

Dihydroberberine was proved to be one of the anti-inflammatory ingredients naturally exited in Phellodendri Chinese Cortex. The anti-inflammatory mechanism might be associated with dual modulation of NF-κB and MAPK signaling pathways and regulation of inflammatory mediators (Fig. 9). The anti-inflammatory effect of dihydroberberine was weaker than that of BBR. This work provides further empirical evidence for the use of Phellodendri Chinese Cortex to treat inflammation-related diseases in folk medicine. Dihydroberberine presumably serves as a lead molecule for further chemical modification to optimize the therapeutic benefit against inflammatory diseases.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

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