PAPER

Inhibitory effects of three bisbenzylisoquinoline alkaloids on lipopolysaccharide-induced microglial activation

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Activation of microglial cells has been considered to be associated with various neurodegenerative diseases. In this study, three bisbenzylisoquinoline alkaloids (liensinine, neferine, and isoliensinine) were found to inhibit microglial activation induced by lipopolysaccharide (LPS). Liensinine, neferine, and isoliensinine suppressed the release of NO and cytokines (TNF-α, IL-1β, and IL-6) by LPS-activated microglial cells. Three alkaloids displayed direct NO-scavenging activity in SNP solution and reduced LPS-induced protein and mRNA expression of iNOS in microglial cells. Further studies revealed that liensinine, neferine, and isoliensinine blocked IκBα phosphorylation and degradation, as well as ROS production in LPS-activated microglial cells. In addition, three alkaloids showed direct scavenging activity against DPPH, ·OH, and ONOO⁻ radicals. These results suggest that liensinine, neferine, and isoliensinine may have potential in the treatment of neurodegenerative diseases accompanied by microglial activation.

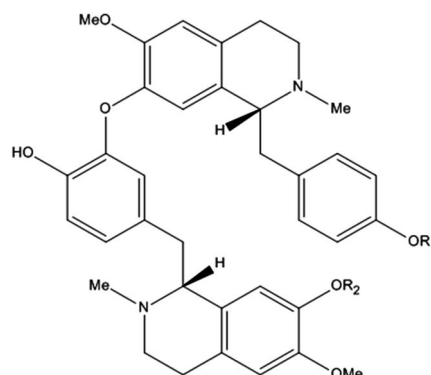## 1 Introduction

Microglial cells are macrophage-like cells, and the first and most important line of immune defense in the central nervous system (CNS).[1] Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) are related to neuroinflammation, featured by the release of neurotoxic factors including nitric oxide (NO) and cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) by microglial cells.[2] The inhibition of microglial activation may lead to the attenuation of the formation and development of many neurodegenerative diseases.[3]

Nelumbo nucifera (lotus) is a well-known edible plant widely cultivated in Asia. Food related to lotus is popular in East Asia, especially China. As a homology of traditional Chinese medicine and food, the green embryo of *Nelumbo nucifera* seeds mainly contain three structure-related bisbenzylisoquinoline alkaloids including liensinine, neferine, and isoliensinine (chemical structures shown in Fig. 1).[4] These three bisbenzylisoquinoline alkaloids are reported to possess a wide range of biological activities including anticarcinogenesis,[5–7] anti-arrhythmia,[8,9] anti-hypertension,[10] sedation,[11] and anti-depression.[12]

Studies have documented that liensinine, neferine, and isoliensinine can cross the blood–brain barrier and have recently received much attention for their neuroprotective effects.[13–15] Liensinine and neferine have been demonstrated to have anti-AD activities by inhibiting acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and β-site amyloid precursor protein-cleaving enzyme 1 (BACE1).[14] In addition, neferine attenuates the protein level and toxicity of mutant huntingtin in PC-12 cells *via* induction of autophagy, which might demonstrate its neuroprotective effect *in vitro*.[15] However, the effects and mechanism of liensinine, neferine, and isoliensinine on the inflammatory activities of microglial cells remain to be unclear.

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R₁=H, R₂=Me: liensinine (1)
R₁=R₂=Me: neferine (2)
R₁=Me, R₂=H: isoliensinine (3)

Fig. 1 Chemical structures of liensinine, neferine, and isoliensinine.

In the present study, the capacity and possible mechanism of liensinine, neferine, and isoliensinine to inhibit microglial activation induced by LPS were investigated.

2 Materials and methods

2.1 Cell culture and drug treatment

Primary microglial cells were prepared from the cortex of newborn Kunming mice (Laboratory Animal Center, China Medical University).[16] All animal experiments were performed following the National Guidelines for Experimental Animal Welfare 78 (MOST, PR China, 2006), and were approved by the Animal Studies Committee of 79 Liaoning University.

Briefly, after the meninges and blood vessels of the mouse cortex were removed, cortical tissue was cut into small pieces and dissociated with 0.25% trypsin–EDTA (Gibco, GrandIsland, NY, USA) at 37 °C for 10 min, and then the cell suspension was filtered through 50 μm diameter nylon mesh. The cells were collected by centrifugation at 1000 rpm for 5 min, re-suspended in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 5% fetal bovine serum (FBS, Gibco), 2 mmol L^{-1} glutamine, penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}), and then plated in culture flasks. Cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2. After 11–14 days, the flasks were shaken by hand for 10 min. The resulting cell suspension, rich in microglial cells, was placed in another flask in which the cells were adhered for 30 min at 37 °C. The purity of microglial cells obtained was >95% as identified by immunocytochemistry using the CD11b monoclonal antibody (ImmunoTools, Friesoythe, Germany).[17]

Liensinine, neferine, and isoliensinine (purity > 98%, Chengdu Mansite Pharmaceutical Co. Ltd, Chengdu, Sichuan, China) were initially dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), and then diluted with DMEM medium without serum. DMSO at the highest concentration possibly present in experimental conditions (0.1%) was not toxic to the cells. Exponentially growing microglial cells were treated with liensinine, neferine, or isoliensinine (0.1–10 μmol L^{-1}) in the presence or absence of LPS (1 μg mL^{-1}, Sigma-Aldrich).

2.2 Cell viability assay

The cell viability was evaluated by the 3-4-5-dimethylthiazole-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) assay. Primary microglial cells were seeded at 4×10^4 cells per well in 96-well plates. After 24 h incubation, the cells were treated with liensinine, neferine, or isoliensinine (0.1–10 μmol L^{-1}) in the presence or absence of LPS (1 μg mL^{-1}) for 48 h. The medium was then removed and the cells were incubated with 0.25 mg mL^{-1} MTT (Gibco) for 4 h at 37 °C. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured at 490 nm by a microplate reader (TECAN, Switzerland).

2.3 Nitrite assay

As a measure of NO release, nitrite accumulation in cell culture supernatant fluids was measured using the Griess reagent.[18]

Microglial cells at a density of 4×10^4 cells per well were seeded in 96-well plates for 24 h and incubated with liensinine, neferine, or isoliensinine (0.1–10 μmol L^{-1}) in the presence or absence of LPS (1 μg mL^{-1}) for 48 h. Fifty microliters of culture supernatant fluids was mixed with an equal volume of the Griess reagent. The Griess reagent was made by a 1 : 1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water. The absorbance of the mixture was measured at 540 nm by the microplate reader. The amount of nitrite was calculated with reference to a sodium nitrite standard curve.

2.4 NO-scavenging activity assay

The NO-scavenging effects of three bisbenzylisoquinoline alkaloids (0.1–10 μmol L^{-1}) were assessed according to the previous method.[18] Sodium nitroprusside (SNP, 2.5 mmol L^{-1}) was incubated alone or combined with various concentrations of liensinine, neferine, or isoliensinine. SNP is an inorganic substance where NO is found as NO^+, and being exposed to light is necessary for the generation of NO. Therefore, the mixtures were incubated in light at room temperature for 60 min, and nitrite levels were detected by the Griess reaction.

2.5 Western blot analysis

Microglial cells cultured in culture flasks were treated with liensinine, neferine, or isoliensinine (1–10 μmol L^{-1}) in the presence of LPS (1 μg mL^{-1}). The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed for 30 min with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) supplemented with 1 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF) immediately before use. Concentration of protein in the supernatant fluids of the lysate was determined by the BCA assay (CWbiotech, Beijing, China) following the manufacturer's protocol. Equal amounts of protein were separated by electrophoresis and then the gel was transferred onto polyvinylidene fluoride membrane (PVDF, CWbiotech). The PVDF membranes were blocked in 5% skimmed milk for 1 h at room temperature and then incubated overnight with primary antibodies against iNOS, IκBα, phosphorylated IκBα (1 : 1000, all from Cell Signaling Technology, Danvers, MA), and β-actin (1 : 1000, Proteintech Group, Wuhan, China), followed by horseradish peroxidase (HRP) conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein of interest was detected by enhanced chemiluminescence (ECL) reagents (Tanon, Shainghai, China) and visualized with a Tanon 5200 Multi Chemiluminescent System (Tanon, Shanghai, China). The relative values for each protein were normalized to β-actin.

2.6 Reverse transcription polymerase chain reaction (RT-PCR) analysis

Microglial cells were placed in 6-well plates and incubated with liensinine, neferine, or isoliensinine (1–10 μmol L^{-1}) in the presence of LPS (1 μg mL^{-1}) for 24 h. Total RNA was extracted with RNAiso plus (TaKaRa Bio Inc., Dalian, China) according to the manufacturer's protocol. The concentrations of total RNA
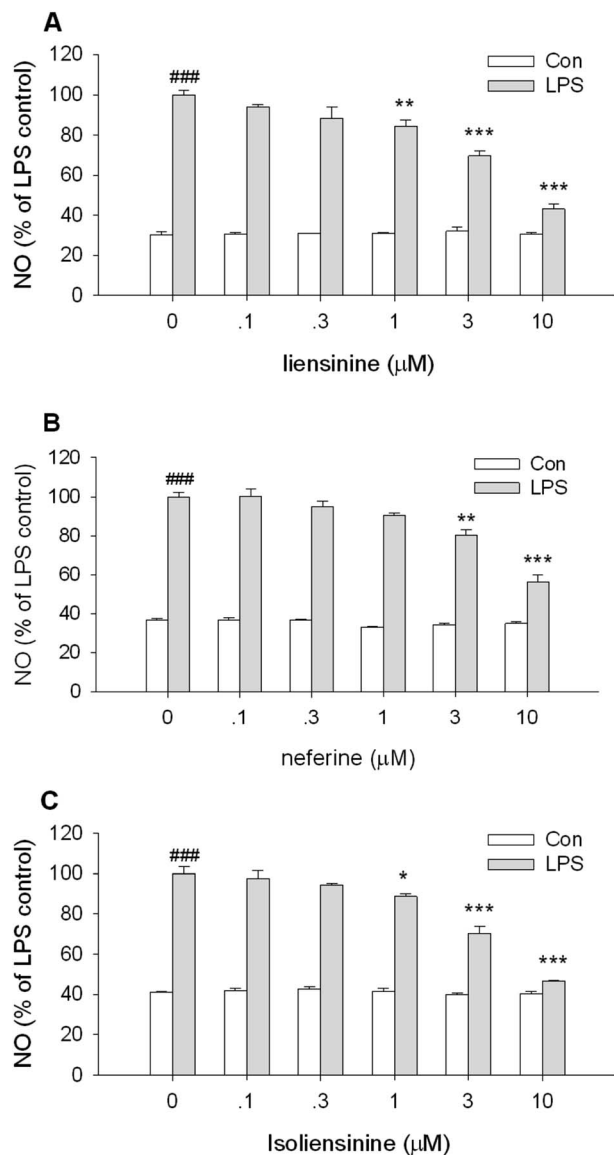
Fig. 2 Inhibitory effects of liensinine, neferine, and isoliensinine on NO production by unstimulated or LPS-activated microglial cells. Primary microglial cells were treated with liensinine (A), neferine (B), or iso-liensinine (C) (0.1–10 μmol L^{-1}) in the presence or absence of LPS (1 μg mL^{-1}) for 48 h. The results were expressed as the percentage values taking LPS treatment group as 100%. Data were represented as mean ± SEM of three separate experiments. $^{###}P < 0.001$ as compared with the control group (cultured in medium alone), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ as compared with LPS alone group.
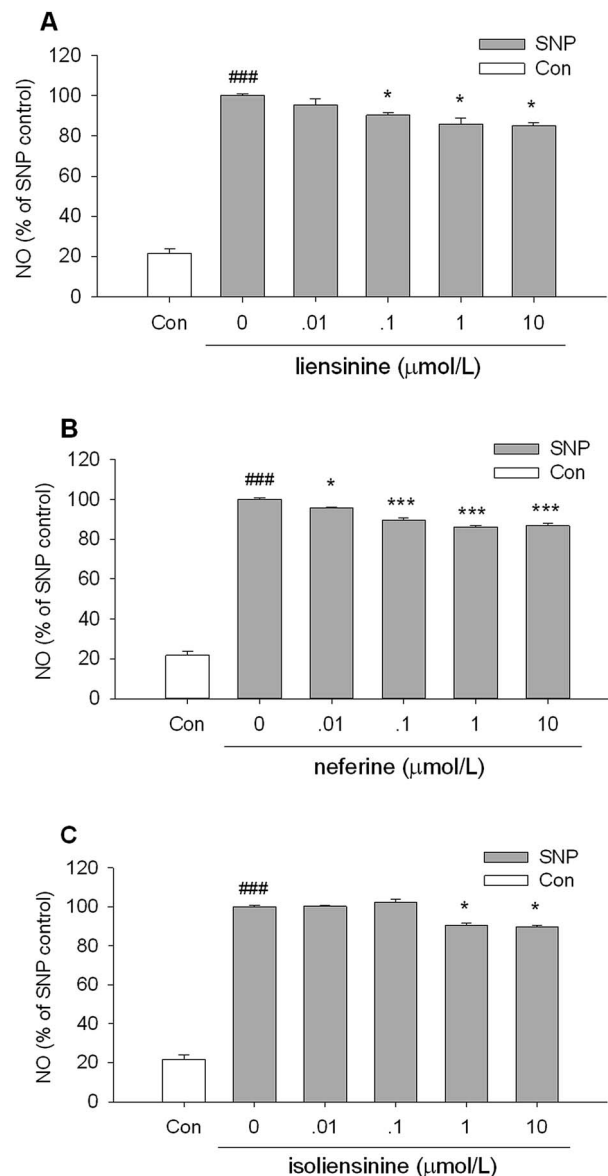

Fig. 3 Effects of liensinine, neferine, and isoliensinine on nitric oxide-scavenging. SNP solution (2.5 mmol L^{-1}) in PBS was incubated alone or with liensinine (A), neferine (B), or isoliensinine (C) (0.01–10 μmol L^{-1}) in light at room temperature for 60 min. The results were expressed as the percentage values taking the SNP group as 100%. Data were represented as mean ± SEM of three separate experiments. $^{###}P < 0.001$ as compared with the control group (PBS alone), $*P < 0.05$, $***P < 0.001$ as compared with the SNP group.

were measured using spectrophotometer. Total RNA (1 μg) was reverse transcribed to complementary DNA (cDNA). In brief, one microgram of total RNA was mixed with oligo(dT) primer (TaKaRa Bio Inc.) and brought to a final volume of 6 μL with RNase-free water. The RNA and primer mixtures were heated at 70 °C for 10 min and then immediately cooled on ice until the reverse transcription mixtures containing 5× M-MLV buffer, dNTP, M-MLV reverse transcriptase, and RNase-free water (all from TaKaRa Bio Inc.) were prepared. A total of 14 μL of the reverse transcription mix and 6 μL of total cellular RNA and

primer mixtures were incubated at 42 °C for 1 h, followed by heating at 70 °C for 15 min. The cDNA was then amplified by PCR using primers specific for the genes of GAPDH and iNOS: GAPDH forward, 5′-AGT GGC AAA GTG GAG ATT GTT G-3′; GAPDH reverse, 5′-CAG TCT TCT GGG TGG CAG TGA T-3′; iNOS forward, 5′-GAC AAG CTG CAT GTG ACA TC-3′; iNOS reverse, 5′-GCT GGT AGG TTC CTG TTG TT-3′. The following conditions of PCR reactions were applied: GAPDH, 28 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s; iNOS, 30 cycles of denaturation at 94 °C for 30 s,
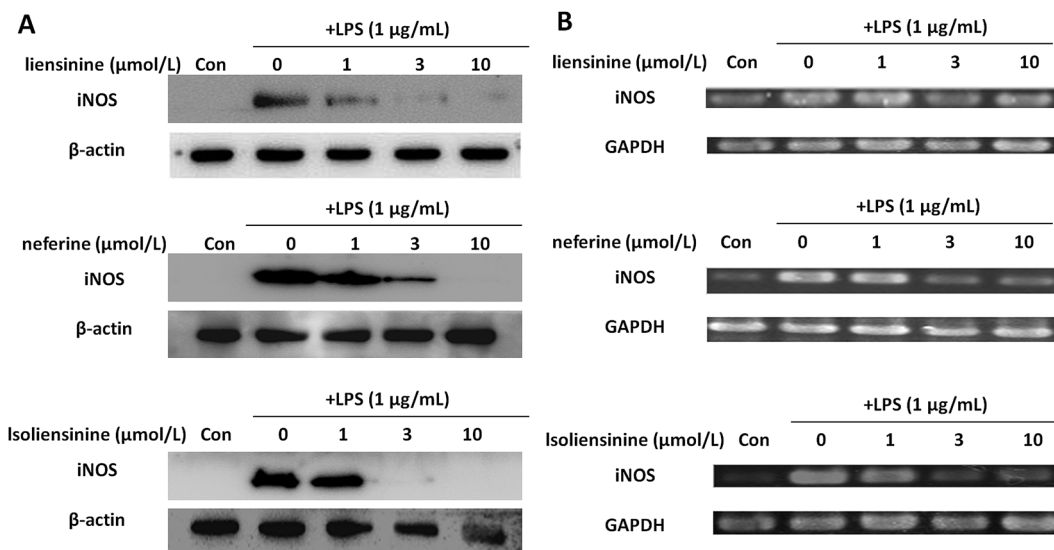
Fig. 4 Inhibitory effects of liensinine, neferine, and isoliensinine on LPS-induced expression of iNOS protein and mRNA in microglial cells. Microglial cells were treated with liensinine, neferine, or isoliensinine (1–10 μmol L^{-1}) in the presence of LPS (1 μg mL^{-1}) for 24 h. (A) The expression of iNOS protein was analyzed by western blot. (B) The expression of iNOS mRNA was detected by RT-PCR.
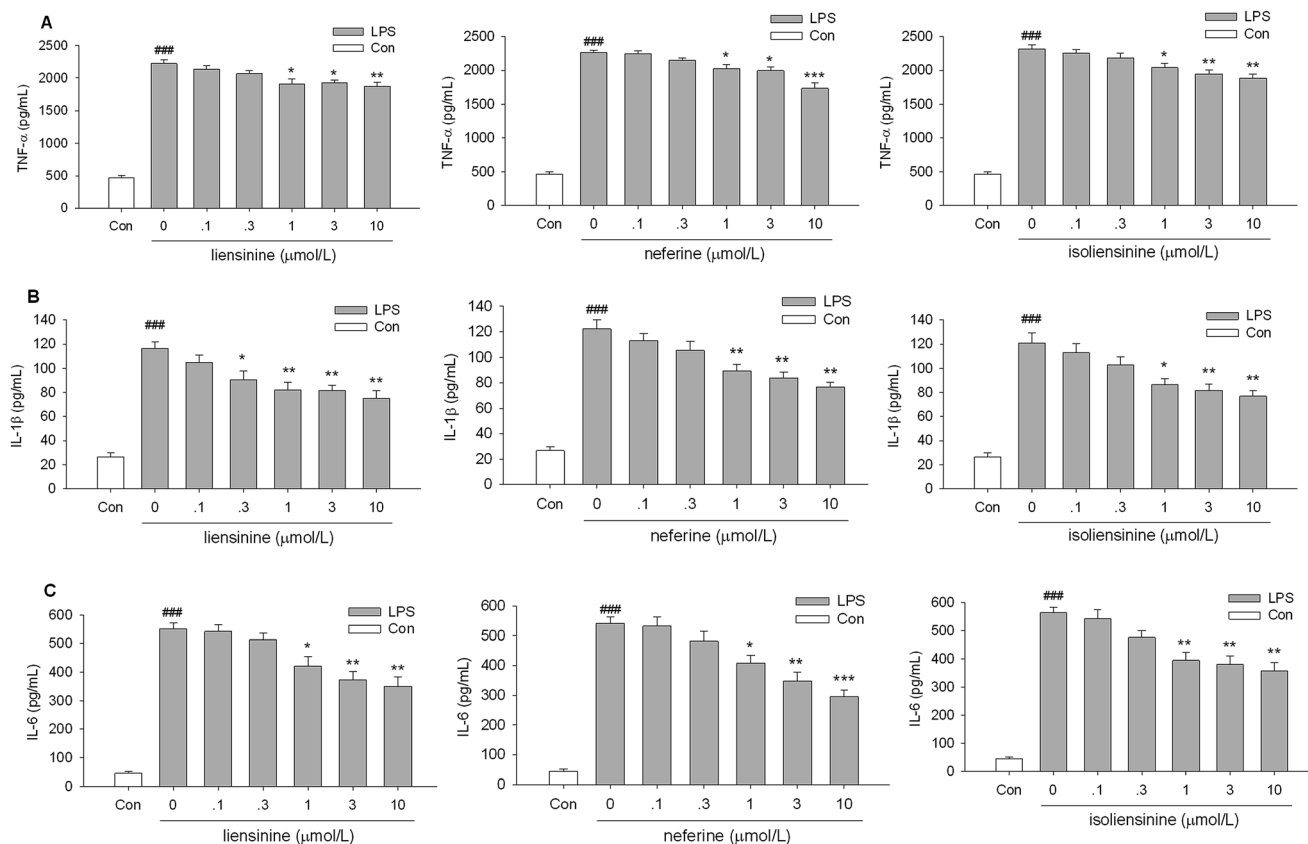

Fig. 5 Inhibitory effects of liensinine, neferine, and isoliensinine on TNF-α, IL-1β, and IL-6 release by LPS-activated microglial cells. Microglial cells were treated with liensinine, neferine, or isoliensinine in the presence of LPS (1 μg mL^{-1}). After incubation for 1 h, the levels of TNF-α (A) in the supernatant fluids were measured. After incubation for 4 h, the levels of IL-1β (B) and IL-6 (C) in the supernatant fluids were measured. Data were represented as mean ± SEM of three separate experiments. $^{###}P < 0.001$ as compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with LPS alone group.

annealing at 58 °C for 30 s, and extension at 72 °C for 30 min. PCR products were separated in 1.2% agarose gels, visualized by staining with ethidium bromide, and yielded the expected amplicons of 480 and 293 bp, respectively.

2.7 Cytokine level measurement

Supernatant fluids of LPS-activated microglial cells incubated with or without liensinine, neferine, or isoliensinine (0.1–10 μmol L^{-1}) were collected to quantitate the production of TNF-α, IL-1β, and IL-6 using the enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.8 Intracellular ROS assay

The intracellular reactive oxygen species (ROS) was detected using 2′,7′-dichlorodihydrofluororescein diacetate (DCFH-DA, Sigma-Aldrich).[19] DCFH-DA was dissolved in methanol at 10 mmol L^{-1} and diluted 500-fold in PBS to give final concentration at 20 μmol L^{-1}. Microglial cells were seeded in 6-well plates for 24 h and then treated with liensinine, neferine, or isoliensininein (1 and 3 μmol L^{-1}) in the presence of LPS (1 μg mL^{-1}) for 12 h. The cells were exposed to DCFH-DA for 30 min at 37 °C. After washing three times with PBS, the cells were resuspended in the PBS. Intracellular ROS levels were analysised by a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The results are presented as mean fluorescence intensity of the population.

2.9 DPPH scavenging activity assay

The DPPH free radical scavenging effects of three compounds were determined according to the previous method.[16] 2,2-Diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich) was dissolved in 100% ethanol at a final concentration of 100 μmol L^{-1}. The mixture was then shaken in the dark for 10 min. Liensinine, neferine, or isoliensininein (500 μL each) was added to 500 μL of DPPH free radical solution. After incubation in the dark at room temperature for 30 min, the absorbance at 517 nm was measured using the microplate reader.

2.10 ·OH scavenging activity assay

Hydroxyl radical (·OH) scavenging effects of three bisbenzylisoquinoline alkaloids were performed as previously described with slight modifications.[20] Liensinine, neferine, or isoliensininein was added to the mixture containing 2 mmol L^{-1} FeSO$_4$ and 0.8 mmol L^{-1} H$_2$O$_2$. After standing at room temperature for 10 min, the reaction was initiated by adding the salicylic acid solution (1.5 mmol L^{-1}). Reaction mixture was incubated at 37 °C for 30 min. The absorbance at 510 nm was detected using the microplate reader.

2.11 ONOO$^-$ scavenging activity assay

The peroxynitrite (ONOO$^-$) scavenging activity was assayed according to the reported method with some modifications.[21] The synthesis reaction was performed at 4 °C. Five milliliters of solution containing 0.08 mL of H$_2$SO$_4$ (96%), 0.3 mL of H$_2$O$_2$

(30%), and 4.62 mL of distilled water was mixed with 5 mL of 0.6 mol L^{-1} NaNO$_2$ at 4 °C. Then, 5 mL of 1.25 mol L^{-1} ice cold NaOH was immediately added to quench the reaction. Residual H$_2$O$_2$ was removed by using 0.08 g MnO$_2$ (solid powder) and the reaction mixture was left overnight at −20 °C in the dark. The absorbance of the complex was detected at 302 nm using a UV spectrophotometer. The concentration of ONOO$^-$ was calculated according to Bouguer–Lambert–Beer law. The L-tyrosine (75 μmol L^{-1}) was mixed with ONOO$^-$ solution (96 μmol L^{-1}), and then liensinine, neferine, or isoliensinine (0.1–10 μmol L^{-1}) was added to the mixture. After incubating at 37 °C in the water bath for 30 min, the absorbance was detected at 428 nm using the microplate reader.

2.12 Statistical analysis

Results are expressed as mean ± SEM for at least three independent experiments. Statistical analysis was performed using SPSS 19.0 software. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test. Significance between two groups was analyzed by t-test.

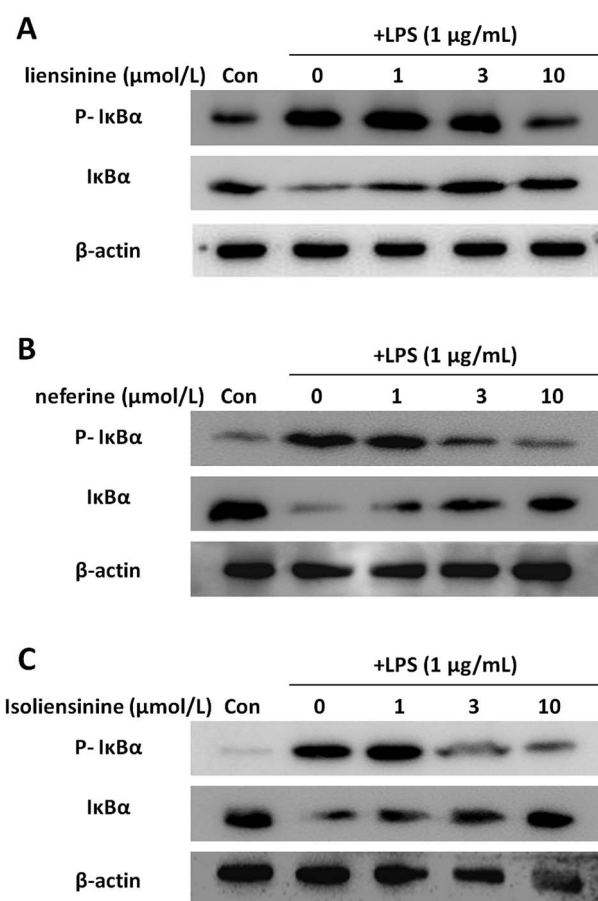Fig. 6 Inhibitory effects of liensinine, neferine, and isoliensinine on IκBα phosphorylation and degradation induced by LPS in microglial cells. Microglial cells were treated with liensinine (A), neferine (B), or isoliensinine (C) in the presence of LPS (1 μg mL^{-1}) for 30 min. Total protein was subjected to 10% SDS-PAGE followed by western blotting using IκBα and phosphorylated IκBα antibodies.

3 Results and discussion

3.1 Inhibitory effects of liensinine, neferine, and isoliensinine on LPS-induced NO production

MTT assay, a kind of indirect cell viability measurement method, is based upon the capability of mitochondrial succinate dehydrogenase in viable cells to deoxygenize MTT to insoluble purple formazan products. In order to avoid the possible effects of reduced viability on NO and cytokine release, the effects of three bisbenzylisoquinoline alkaloids on the cell viability were studied at first. Co-treatment of either unstimulated or stimulated primary microglia with liensinine, neferine, or isoliensinine (0.1–10 μmol L^{-1}) did not affect the viability of microglial cells (data not shown). The results indicate that the effects of three alkaloids on microglial cells were unlikely due to the toxicity. However, it is widely known that most of alkaloids have a high toxicity *in vivo*, further studies are required in order to understand the safety of three alkaloids *in vivo*.

Nitric oxide (NO), synthesized by a conversion of L-arginine to L-citrulline by iNOS, is an important mediator and its generation is a crucial procedure in the activation of microglia.[22] A significant enhancement in the expression of iNOS in microglial cells leads to surplus production of NO, which associated with various neurodegenerative diseases.[23] In this study, the production of NO by LPS-stimulated microglial cells was examined. As shown in Fig. 2, LPS caused a substantial increase in NO production compared to the control group ($P < 0.001$). Three bisbenzylisoquinoline alkaloids inhibited LPS-induced NO production in a concentration-dependent manner, with the IC$_{50}$ values of 4.4, 6.9, and 3.8 μmol L^{-1}, respectively. The results presented here indicated that liensinine and isoliensinine had more potent inhibitory activity on NO production than neferine. As a bisbenzylisoquinoline alkaloid originated from the dried root of one Chinese herb of Radix *Stephania tetrandra*, tetrandrine has been proved to exert the anti-inflammatory effect by inhibiting the production of NO in activated microglial cells induced by LPS or Amyloid-β (Aβ).[24,25] In this study, the effect of tetrandrine on NO production in activated microglia was also observed. The results showed that liensinine, neferine, and isoliensinine had more potential inhibitory effect on NO production than that of tetrandrine (IC$_{50}$ = 7.9 μmol L^{-1}; data not shown). Furthermore, treatment of unstimulated cells with three alkaloids (0.1–10 μmol L^{-1}) did not result in any change of NO production.
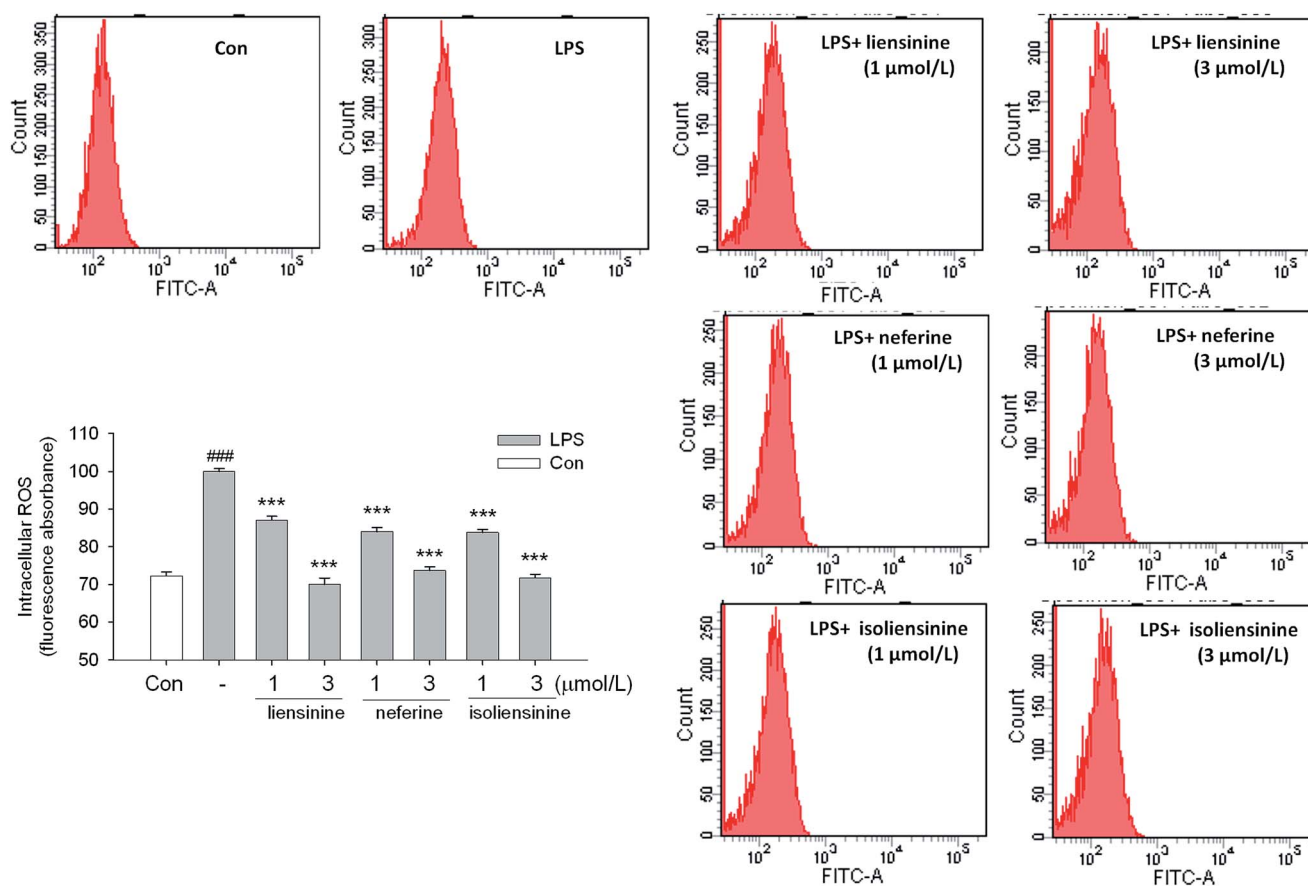

Fig. 7 Inhibitory effects of liensinine, neferine, and isoliensinine on LPS-induced ROS production in microglial cells. Microglial cells were treated with liensinine, neferine, or isoliensinine (1 and 3 μM) in the presence of LPS (1 μg mL^{-1}) for 12 h. Intracellular ROS were determined by DCFH oxidation. The results were expressed as the percentage values taking LPS treatment group as 100%. Data were represented as mean ± SEM of three separate experiments. $^{###}P < 0.001$ as compared with the control group, $^{***}P < 0.001$ as compared with LPS alone group.

3.2 NO-scavenging effects of liensinine, neferine, and isoliensinine

Then, the NO-scavenging activity of three bisbenzylisoquinoline alkaloids was measured using SNP as a NO donor to explore whether the suppressive effects of NO release were attributed to direct NO-scavenging activity.[26,27]

As shown in Fig. 3, when a solution of 2.5 mmol L^{-1} SNP was incubated in light at room temperature for 1 h, it generated nitrite which was decreased by the presence of liensinine (Fig. 3A), neferine (Fig. 3B), or isoliensinine (Fig. 3C) in a concentration-dependent manner. Three bisbenzylisoquinoline alkaloids displayed low direct NO-scavenging activity and might act as the potent NO inhibitors in microglial cells. The NO-scavenging activity of neferine was consistent with the previous report.[13] Moreover, the direct NO-scavenging effects of three alkaloids are weaker than that of NO production by LPS-activated microglial cells (Fig. 2), which indicated that the direct NO-scavenging effects are likely to be one of the mechanisms of these alkaloids to decrease the NO level in culture supernatant fluids of LPS-activated microglial cells.

3.3 Effects of liensinine, neferine, and isoliensinine on LPS-induced expression of iNOS protein and mRNA

In order to evaluate whether the suppression of three alkaloids on NO generation was related to the regulation of iNOS induction, we detected iNOS protein and mRNA expression in LPS-activated microglia. As shown in Fig. 4, the levels of iNOS protein and mRNA expression in microglial cells were markedly up-regulated after LPS stimulation for 24 h. Co-incubation of the microglial cells with LPS plus liensinine, neferine, or isoliensinine (1–10 μmol L^{-1}), the expression of iNOS in the cells was concentration-dependently suppressed at both the protein levels (Fig. 4A) and mRNA levels (Fig. 4B). Moreover, the mRNA expression levels of iNOS followed the same trends as those of the protein. Therefore, the decrease of NO production may be attributed to the inhibition of iNOS mRNA transcription followed by reduced iNOS protein production, and direct NO-scavenging effects mentioned above. In addition, liensinine and isoliensinine were found to have more potent inhibitory effects on iNOS protein production than neferine.

3.4 Inhibitory effects of liensinine, neferine, and isoliensinine on LPS-induced TNF-α, IL-1β, and IL-6 release

TNF-α, IL-1β, and IL-6 are key inflammatory cytokines, which have been verified to be responsible for inflammation accompanied by the brain damage.[28,29] The levels of three activation-related cytokines (TNF-α, IL-1β, and IL-6) in cell culture supernatant fluids were measured by ELISA. Stimulation of microglial cells with LPS led to an increase in TNF-α, IL-1β, and IL-6 levels in the culture media. Liensinine, neferine, and isoliensinine could significantly inhibit LPS-induced TNF-α (Fig. 5A), IL-1β (Fig. 5B), or IL-6 release (Fig. 5C) at the concentrations of 1, 3, and 10 μmol L^{-1}. These results further
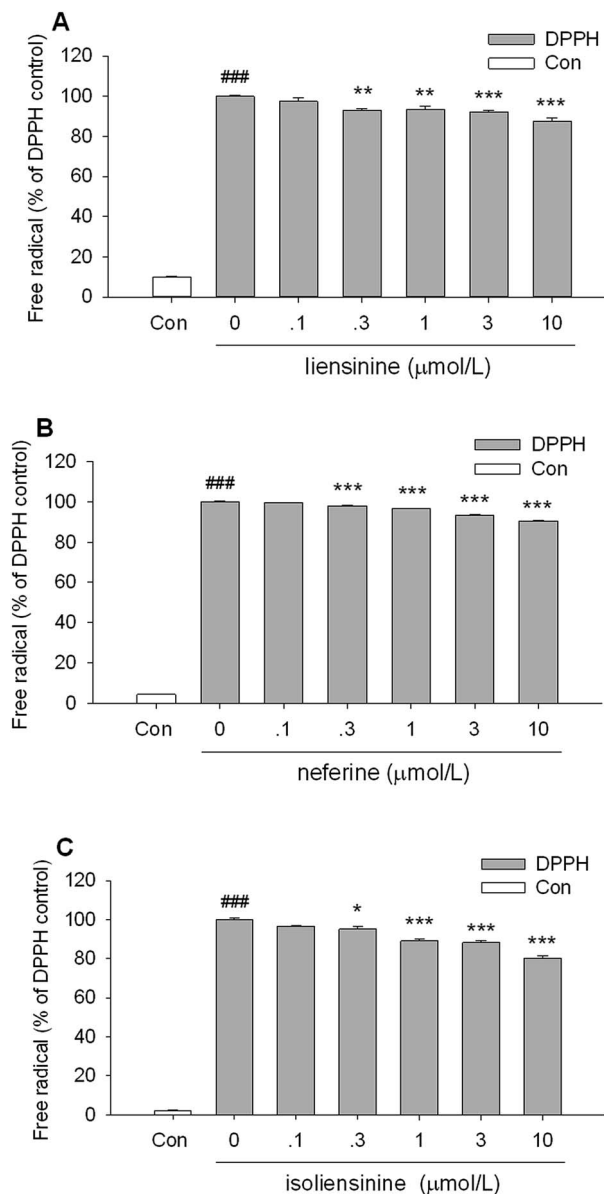

Fig. 8 DPPH free radical scavenging activity of liensinine, neferine, and isoliensinine. The results were expressed as the percentage values taking only DPPH group as 100%. Data were represented as mean ± SEM of three separate experiments. $^{###}P < 0.001$ as compared with the control group (100% ethanol alone), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ as compared with only DPPH group.

confirmed the inhibitory effects of three alkaloids on microglial activation.

However, it should be noticed that three alkaloids were more effective on NO production than on TNF-α, IL-1β, and IL-6 release, which suggested that the regulatory mechanisms of three alkaloids on TNF-α, IL-1β, IL-6, and NO production may be at least partly different. Liensinine, neferine, and isoliensinine have also been demonstrated to blockade of Ca^{2+} influx.[30–32] Whether the Ca^{2+} signaling pathway contributes to the inhibition of NO and cytokines production by three alkaloids in LPS-activated microglia will
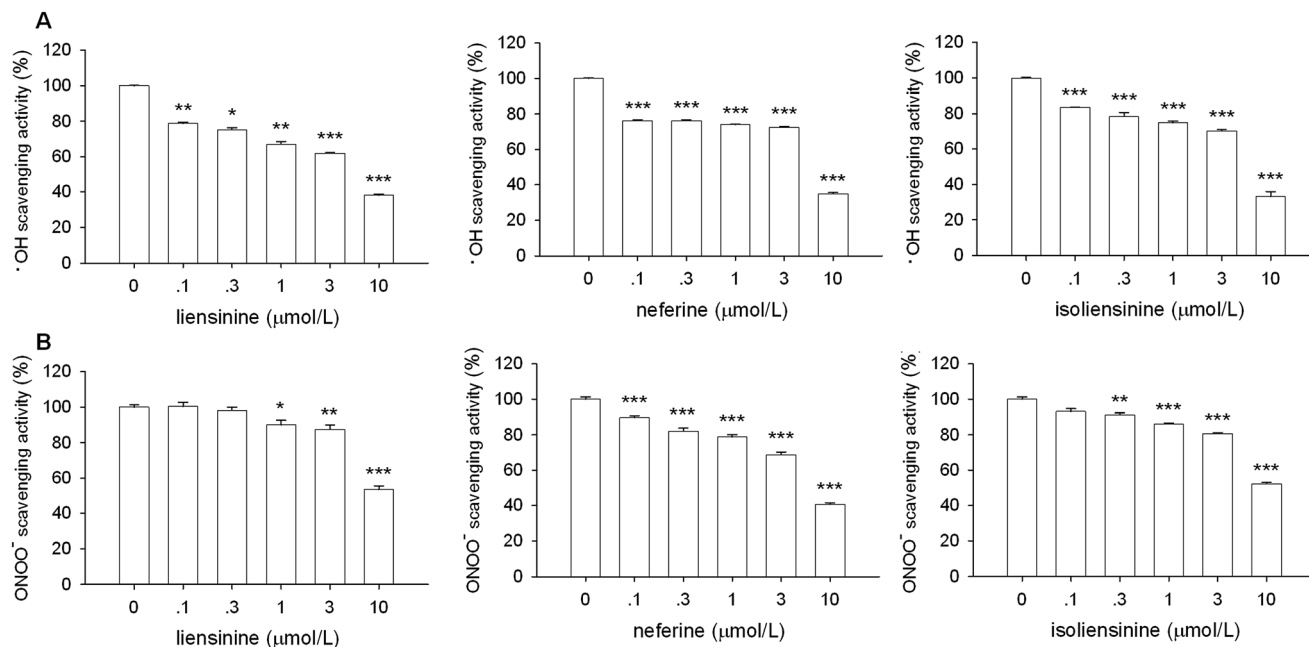
Fig. 9 ·OH and ONOO⁻ free radical scavenging activity of liensinine, neferine, and isoliensinine. The results were expressed as the percentage values taking model group as 100%. Data were represented as mean ± SEM of three separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 as compared with model group.

be further investigated. The exact mechanism remains to be clarified.

3.5 Inhibitory effects of liensinine, neferine, and isoliensinine on LPS-induced IκBα phosphorylation and degradation

NF-κB is considered to be a major transcription factor in the regulation of the inflammatory cytokines and inducible enzymes, such as TNF-α, IL-1β, IL-6, and iNOS.[33] In resting cells, NF-κB is located in the cytoplasm as an inactive complex bound to its inhibitor, IκB. The activation of NF-κB depends on phosphorylation, ubiquitination, and subsequent degradation of IκB protein; IκBα is the most well characterized.[34] The liberated NF-κB translocates to the nucleus and binds to the regulatory region in the promoter of specific genes, leading to their transcription.[35] Isoliensinine has been found to intervene in NF-κB activation in hepatocellular carcinoma cells.[6,36] Neferine was shown to inhibit NF-κB activation and nuclear translocation in lung cancer cells,[37] human rhabdomyosarcoma cells,[38] vascular smooth muscle cells,[39] and endothelial cells.[40] However, the effects of liensinine, neferine, and isoliensinine on the NF-κB activation of microglial cells remain to be unclear.

In this study, the levels of p-IκBα were increased and the levels of IκBα were decreased after LPS treatment, indicating IκBα was prominently phosphorylated and degraded (Fig. 6). Liensinine (Fig. 6A), neferine (Fig. 6B), or isoliensinine (Fig. 6C) treatment resulted in the inhibition of LPS-induced phosphorylation and degradation of IκBα protein. These findings demonstrated that three alkaloids exerted their inhibitory effects on LPS-induced microglial activation by blocking the NF-κB signaling pathway.

3.6 Inhibitory effects of liensinine, neferine, and isoliensinine on LPS-induced ROS production

Activated microglial cells produce high levels of ROS by activating NADPH oxidase (NOX),[41,42] and ROS may be an early signal triggering the induction of inflammatory cytokines and participating in inflammatory signaling pathways.[43] Moreover, intracellular redox changes induced by ROS augment NF-κB activation through the phosphorylation and degradation of IκB via increasing Akt or IKK kinase activity.[44] Thus, ROS/NF-κB signaling pathway may play a pivotal role in LPS-activated microglial cells.[45]

In our study, the results showed that liensinine, neferine, and isoliensinine (1 and 3 μmol L⁻¹) effectively suppressed intracellular ROS production in LPS-stimulated microglial cells, which suggested that they are potent antioxidants (Fig. 7).

3.7 DPPH, ·OH, and ONOO⁻ scavenging effects of liensinine, neferine, and isoliensinine

As shown in Fig. 8, all of the three bisbenzylisoquinoline alkaloids showed low direct scavenging effects on the artificial model radical DPPH in a cell free assay. The scavenging effects of three alkaloids on DPPH radicals appeared at the concentrations of 0.3–10 μmol L⁻¹. The DPPH free radicals scavenging effects of three alkaloids are weaker than that of intracellular ROS production by LPS-activated microglial cells (Fig. 7), which suggested that the free radicals scavenging effects are likely to be one of the mechanisms of these alkaloids to decrease the intracellular ROS level in LPS-activated microglial cells.

As shown in Fig. 9A, liensinine, neferine, and isoliensinine showed potent scavenging activities against ·OH radicals as compared with the model group, with the IC_{50} values of 5.4, 6.9, and 6.6 μmol L^{-1}, respectively. In addition, liensinine, neferine, and isoliensinine exhibited significant scavenging activities against $ONOO^-$ in a concentration-dependent manner, with respective IC_{50} values of 10.5, 7.8, and 10.3 μmol L^{-1} (Fig. 9B).

Three alkaloids exerted scavenging activities against DPPH, ·OH, and $ONOO^-$ radicals. These results suggest that liensinine, neferine, and isoliensinine may suppress NF-κB signaling at least partly by scavenging the free radicals and inhibiting the ROS generation in LPS-activated microglial cells.

4 Conclusion

In summary, these findings demonstrate for the first time that liensinine, neferine and isoliensinine exert anti-neuroinflammatory activity by suppressing the production of NO, TNF-α, IL-1β, and IL-6 in LPS-activated microglial cells. Moreover, three bisbenzylisoquinoline alkaloids inhibit the phosphorylation and degradation of IκBα induced by LPS in microglial cells, and this effect is mediated at least in part by inhibiting the intracellular ROS generation and scavenging the free radicals.

Our results revealed the key roles of liensinine, neferine and isoliensinine in the anti-neuroinflammatory effects of the embryo of *Nelumbo nucifera* seeds and open a new avenue to understand the potential of this health food in the treatment of neurodegenerative diseases accompanied by microglial activation.

Acknowledgements

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