Lucyoside B, a triterpenoid saponin from *Luffa cylindrica*, inhibits the production of inflammatory mediators via both nuclear factor-κB and activator protein-1 pathways in activated macrophages

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ABSTRACT

Lucyoside B is one of the major triterpenoid saponins in the fruit of *Luffa cylindrica*. Although some chemical and bioactive studies on the extracts of *Luffa cylindrica* have been reported, pharmacological action of lucyoside B remains obscure. In this study, we explored the anti-inflammatory activity of lucyoside B in activated macrophages. Our data showed that lucyoside B suppressed the proinflammatory mediators, such as iNOS, IL-6 and MCP-1, at the transcriptional and translational levels, as well as the production of NO, indicating the potent anti-inflammatory effect in lipopolysaccharide-primed macrophages. Mechanistically, lucyoside B inhibited the phosphorylation and degradation of IκBα and thus prevented the nuclear translocation of p65 to suppress NF-κB transcriptional activity. Concurrently, it also decreased JNK1/2, ERK1/2 and p38 phosphorylation to reduce the transcriptional activity of AP-1. Our study suggests that lucyoside B may be a beneficial food constituent for inflammatory diseases.

1. Introduction

Macrophages play a central role in host defense against pathogen microbes. By recognizing bacterial components, macrophages activate an arsenal of anti-bactericidal effectors and initiate the inflammatory cascades (Fontaine et al., 2007). Lipopolysaccharide (LPS), a major component of the outer membrane in Gram-negative bacteria, is regarded as a pathogen-associated molecular pattern, and can be recognized by toll-like receptor-4 (TLR4) on the membrane of macrophages (Poltorak et al., 1998). The activation of TLR4 signaling by LPS results in the over-production of pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) (Adams and Hamilton, 1984), which causes inflammation and damages neighboring tissues and the macrophages themselves (Tiwari, Dwivedi, & Kakkar, 2010). Thus, inhibition of the over-production of these inflammatory mediators may be a useful therapeutic strategy for treating inflammation-related diseases (Nam, 2006; Wang, Chen, Chen, Chang, & Lee, 2007).

Stimulation of TLR4 by LPS triggers the activation of two early transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which are vital in the regulation of inflammatory responses (Adcock, 1997). NF-κB is a classical and key dimeric transcription factor which controls the transcription of many inflammatory-related genes (Ghosh, May, & Kopp, 1998). At baseline, inhibitor of nuclear factor κB-α (IκBα) regulates NF-κB by binding with and trapping it in the cytoplasm. LPS stimulation activates inhibitor of nuclear factor κB kinase β that phosphorylates IκBα and leads to the degradation of IκBα, thus freeing NF-κB p50/p65 heterodimer to enter the nucleus (Zandi, Chen, & Karin, 1998). Meanwhile, LPS also leads to the activation of three functional parallel mitogen-activated protein kinases (MAPKs) (Nishida & Gotoh, 1993), including c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), extracellular signal-regulated kinase (ERK), and p38 MAPK (Rao, 2001). MAPKs phosphorylation ultimately causes AP-1 translocating into the nucleus to induce the transcription of inflammatory genes (Yang, Sharrocks, & Whitmarsh, 2013). Therefore, blocking either the NF-κB or AP-1 signaling pathway, or both, is a

promising approach for the treatment of inflammatory events (Endale et al., 2017).

In the tropics and subtropics of Asia, *Luffa cylindrica* is grown as an edible vegetable for its fresh fruit. Moreover, the ripe dried vascular bundles of *Luffa cylindrica* fruits, called *Fructus Retinervus Luffae* (Sigualuo), are traditionally used in folk Chinese medicine for the treatment of inflammation related diseases, such as rheumatism (Partap, Kumar, Sharma, & Jha, 2012; Wagner, Bauer, Melchart, Xiao, & Staudinger, 2009), bronchitis and asthma (Simeon, Imeh, & Gbola, 2017). Pharmacological studies also showed that the extracts of different parts of *Luffa cylindrica* fruit were able to exert anti-inflammatory effects. For example, the fruit peel extract inhibited the paw edema caused by carrageenan in rat (Abirami, Indhumathy, Devi, & Kumar, 2011; Kanwal, Syed, Salman, & Mohtasheem, 2013), the fruit pulp extract suppressed the Dermatophagoides farinae-induced atopic dermatitis-like skin lesions in mice (Ha et al., 2015), and the seed extract exerted anti-inflammatory activity in carrageenan induced rat paw-edema (Muthumani et al., 2010). *In vitro* studies also indicated that the extract of *Luffa cylindrica* fruits inhibited the expression of pro-inflammatory mediators in LPS-primed RAW264.7 macrophages (Bor, Chen, & Yen, 2006; Kao, Huang, & Chen, 2012). These researches indicated that *Luffa cylindrica* is a beneficial medicinal and edible plant for inflammatory diseases. As the major effective constituents in the *Luffa cylindrica* fruit, triterpenoid saponins also have multiple activities including anti-stress, immunoregulation (Du & Li, 2005) and the enhancement of macrophage phagocytosis (He, Liu, & Qi, 1997). Lucyoside B (Fig. 1), firstly isolated and identified by Takemoto et al. in 1984 (Takemoto et al., 1984), is a major triterpenoid saponin in *Luffa cylindrica* fruit (Kulkarni, Bhalke, Pande, & Kendre, 2014; Partap, Kumar, Sharma, & Jha, 2012; Tang & Eisenbrand, 1992). Although the content of lucyoside B in *Luffa cylindrica* fruit has not been reported, its proportion in the total triterpenoid saponins from dried leaves and stem was found to be approximately 0.3% (Takemoto et al., 1984). Little research focuses on the pharmacological activity of lucyoside B. In the present study, we investigated the anti-inflammatory activity and the underlying mechanisms of lucyoside B in activated macrophages.

2. Materials and methods

2.1. Chemicals and regents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were produced by Gibco BRL (Grand Island, NY, USA). Lucyoside B was purchased from Push Bio-technology Co. (Chengdu, China) and dissolved in DMSO at the concentration of 50 mM. The recombinant mouse macrophage colony-stimulating factor (M-CSF) protein was from Sino Biological Inc (Beijing, China). Mouse IL-6 and MCP-1 ELISA kits were obtained from Biolegend Co. (San Diego, CA, USA). Antibodies for iNOS and NF-κB p65 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). Antibodies against phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK/SAPK and JNK/SAPK were obtained from Cell Signaling Technology (Danvers, CO, USA). Antibodies against Histone H3, β-Actin and horseradish peroxidase (HRP)-conjugated anti-rabbit or
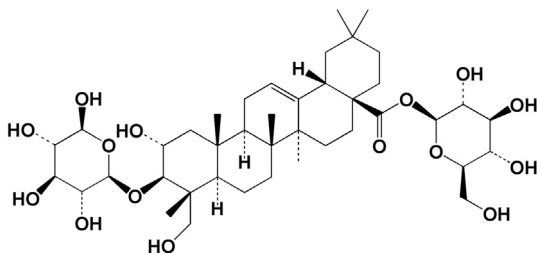

Fig. 1. Chemical structure of lucyoside B.

mouse IgG secondary antibodies were obtained from ABclonal Biotech Co. (Wuhan, Hubei, China). Antibodies against phospho-IκBα, IκBα and the plasmids for NF-κB-TA-luc, AP-1-TA-luc and luciferase assay system were from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). LPS and lipoteichoic acid (LTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Entranstera™-H4000 reagent was purchased from Engreen Biotechnology Co. (Beijing, China). All other reagents were of analytical grade.

2.2. Cell isolation, culture and treatment

The murine macrophage RAW264.7 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin in a humidified incubator with 5% CO_2 at 37 °C. Bone marrow-derived macrophages (BMDMs) were generated from 8 to 12 weeks male BALB/c mice which were purchased from Vital River Experimental Animal Services (Beijing, China). The BMDMs were isolated and cultured according to the procedure previously described (Celada, Gray, Rinderknecht, & Schreiber, 1984). Briefly, the mice were euthanized and both femurs were dissected free of adherent tissue. The ends of the bones were cut off and the marrow tissue were eluted by irrigation with approximate 1 mL DMEM. After being washed, the cells were cultured in DMEM complete medium containing 100 ng/mL M-CSF with replacement of fresh culture medium every 24 h. Seven days later, macrophages that were loosely adherent to the dishes were harvested with cold PBS. When performing treatment, 25, 50 or 100 μM of lucyoside B were first added to the culture medium and then mixed thoroughly (the final concentrations of DMSO were ≤0.25%).

The animal use procedure was approved by the Institutional Animal Care and Use Committee of the Institute of Medicinal Plant Development.

2.3. Measurement of nitrite

The level of nitric oxide (NO) in the culture medium was measured by the Griess method. Cells were seeded at a density of 4×10^5 cells per well in 96-well plates. After pretreated with lucyoside B at 25, 50 or 100 μM for 2 h, 5 ng/mL of LPS or 1 μg/mL of LTA was added to the medium for further 24 h incubation. Nitrite production was measured by mixing 100 μL of supernatant and 100 μL of Griess reagent [0.1% (w/v) N-(1-naphthyl)-ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid] in a 96-well plate. Optical density at 540 nm was measured with a microplate reader. The NO concentration was calculated from a sodium nitrite standard curve.

2.4. iNOS activity assay

The activity of iNOS was assayed as previously described with slight modifications (Chen et al., 2001). The cells were cultured in a 75 cm² flask and incubated with LPS (5 ng/mL) for 12 h to induce intracellular iNOS formation. Then LPS was withdrawn from the cells by washing 3 times to avoid the continuous expression of iNOS, and the cells was harvested and plated in a 48-well plate at a density of 8×10^5 cells per well with lucyoside B (25, 50 or 100 μM) for another 12 h incubation. The nitrite level in the supernatant was assayed by the Griess method to represent the iNOS activity.

2.5. Measurement of IL-6 and MCP-1

Cells were seeded at a density of 4×10^5 cells per well in 96-well plates. After the cells were pretreated with lucyoside B at different concentrations (25, 50 or 100 μM) for 2 h, LPS (5 ng/mL) was added to the medium for another 24 h. Then, the supernatant was obtained for IL-6 and MCP-1 measurement using ELISA kits according to the

Table 1
Primers used for qPCR analysis.

Target gene	Primer sequence
iNOS	Fw: 5′-CTCAGCCCAACAATACAAG-3′
	Rv: 5′-CTACAGTTCCGAGCGTCA-3′
IL-6	Fw: 5′-CGATAGTCAATTCCAGAAACCGC-3′
	Rv: 5′-TTGGGAGTGGTATCCTCTGTGAAG-3′
MCP-1	Fw: 5′-CATCCACGTGTTGGCTCA-3′
	Rv: 5′-GATCATCTTGCTGGTGAATGAGT-3′
β-Actin	Fw: 5′-TGTTACCAACTGGGACGACA-3′
	Rv: 5′-AAGGAAGGCTGGAAAAGAGC-3′

manufacturer's instructions. The concentrations of IL-6 and MCP-1 were calculated from their standard curves, respectively.

2.6. RNA extraction and quantitative real-time PCR

RNA extraction and quantitative real-time PCR (qPCR) assays were performed as previously described (Hou et al., 2018). Briefly, RAW264.7 macrophages were seeded at a density of 1×10^7 cells per well in 6-well plates. The cells were pretreated with 25, 50 or 100 µM of lucyoside B for 2 h and then exposed to LPS (5 ng/mL) for 4 h. Then, total mRNA was extracted. The levels of iNOS, IL-6 and MCP-1 mRNA were normalized to β-Actin. The primers used in these experiments were shown in Table 1.

2.7. Western blot analysis

The levels of iNOS, pIκBα, IκBα, cytoplasmic p65 along with the corresponding β-Actin for every protein, and nuclear p65 with Histone-H3, p-JNK with JNK, p-ERK with ERK, p-p38 with p38 were determined by western blot analysis.

After various treatments, total or cytoplasmic or nuclear proteins were extracted and 30 µg per lane of the proteins were separated by SDS-PAGE, and then were transferred to polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 2 h with 5.0% nonfat milk and then incubated with each primary antibody at 4 °C overnight. After being washed for 3 times, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The blots were visualized using the Gel Doc EQ System (Bio-Rad, Hercules, USA) and the optical density of the bands were quantified by Image J software (Stuttgart, Baden-Württemberg, Germany).

2.8. Luciferase reporter assay

RAW264.7 macrophages were transfected with the luciferase reporter plasmids pAP-1-TA-luc and pNF-κB-TA-luc using Entranstera™-H4000 reagent. Twenty-four hours h after transfection, the cells were

pretreated with 25, 50 or 100 µM of lucyoside B for 2 h and then exposed to LPS (5 ng/mL) for 4 h. Then the cells were lysed and luciferase activity was measured using the Luciferase Assay System.

2.9. Statistical analysis

The data were calculated as the mean ± SD from at least three independent experiments. The Student's t-test was performed to compare two groups. Analysis of variance (ANOVA) was used when more than two groups were compared, and following a statistically significant ANOVA, between-group comparisons were performed using Tukey's post hoc analysis. The analyses were performed using GraphPad Prism 7.0 Software (La Jolla, CA, USA). Difference was accepted at $P < 0.05$.

3. Results

3.1. Lucyoside B inhibits NO production in LPS-primed macrophages

The cell viability analysis showed that lucyoside B exerted no toxic effect up to a concentration of 100 µM (data not shown). We first measured the effect of lucyoside B on NO production in LPS-primed RAW264.7 and BMDMs in a safe concentration range. As shown in Fig. 2A and B, lucyoside B inhibited LPS-induced NO production in a concentration-dependent manner. As in macrophages, NO is produced by an inducible enzyme iNOS that expresses only when exposed to stimulators (Vodovotz, Bogdan, Paik, Xie, & Nathan, 1993). In order to assay the effect of lucyoside B on iNOS activity in a cell-system, RAW274.7 cells were treated with LPS for 12 h to induce the expression of iNOS and then the LPS was removed. In this context, the amount of iNOS protein inside the cells was fixed, and the NO production only represented the change of iNOS activity, rather than its quantity. By measuring the amount of NO in medium, it was found that lucyoside B did not affect iNOS activity (Fig. 2C).

3.2. Lucyoside B suppresses iNOS at transcriptional and translational levels

Owing to the fact that lucyoside B restrained NO production without affecting iNOS activity, we next investigated the inhibitory effects of lucyoside B on iNOS mRNA and protein levels. Our data showed that LPS stimulation of RAW264.7 macrophages resulted in a significant increase in iNOS at the transcriptional and translational levels, while lucyoside B inhibited LPS-induced iNOS mRNA and protein expressions (Fig. 3).

3.3. Lucyoside B suppresses LPS-induced IL-6 and MCP-1 at transcriptional and translational levels

The effect of lucyoside B on the production of pro-inflammatory cytokines IL-6 and MCP-1 was examined. As shown in Fig. 4A and B,
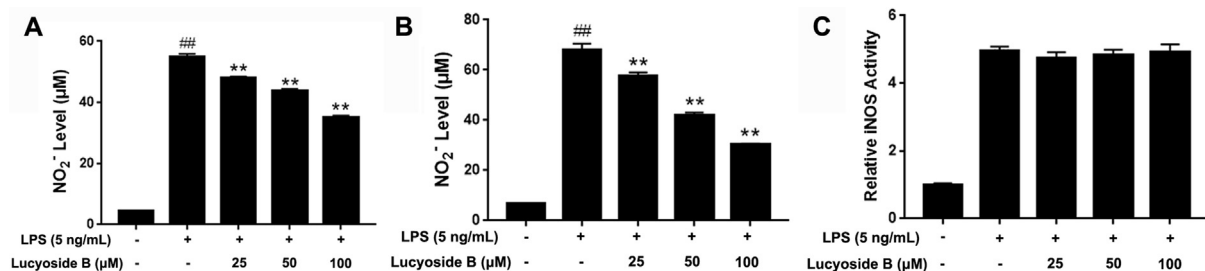

Fig. 2. Effects of lucyoside B on NO production and iNOS activity. (A-B) Effect of lucyoside B on LPS-induced NO production in RAW264.7 macrophages (A) and BMDMs (B) (n = 3). Cells were pretreated with lucyoside B at the indicated concentrations for 2 h and then exposed to LPS (5 ng/mL) for 24 h. Nitrite levels in the culture medium were measured by the Griess reaction. (C) Effect of lucyoside B on iNOS activity (n = 3). Cells were pretreated with LPS (5 ng/mL) for 12 h, LPS was removed by washing, and the cells were treated with lucyoside B for an additional 12 h. Nitrite levels in the culture medium were measured. $^{##}P < 0.01$ compared with the normal control group; $^{**}P < 0.01$ compared with the LPS-treated model group.
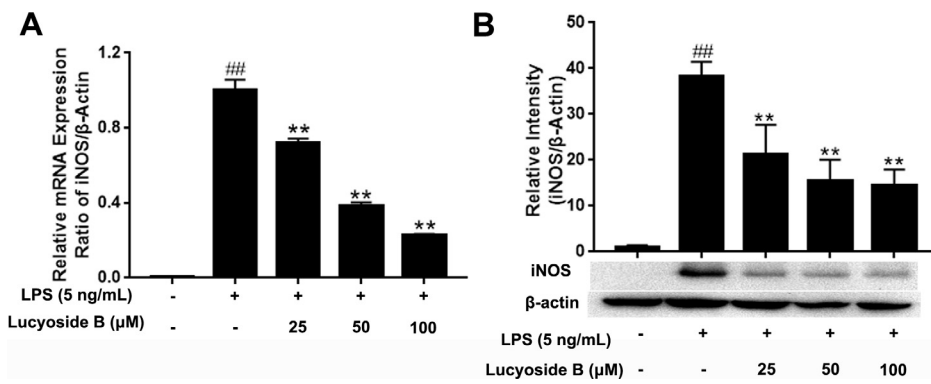
Fig. 3. Effects of lucyoside B on iNOS mRNA and protein expressions in LPS-activated RAW264.7 macrophages. (A) Effect of lucyoside B on iNOS mRNA expression (n = 3). The cells were pretreated with lucyoside B for 2 h and then exposed to LPS (5 ng/mL) for 4 h. Total mRNA was extracted, and the mRNA expression of iNOS was determined by qPCR analysis. (B) Effect of lucyoside B on iNOS protein levels (n = 3). Cells were pretreated with lucyoside B for 2 h and then exposed to LPS (5 ng/mL) for 24 h. The protein level of iNOS was determined by western blot analysis. $^{\#\#}P < 0.01$ compared with the normal control group; $^{**}P < 0.01$ compared with the LPS-treated model group.
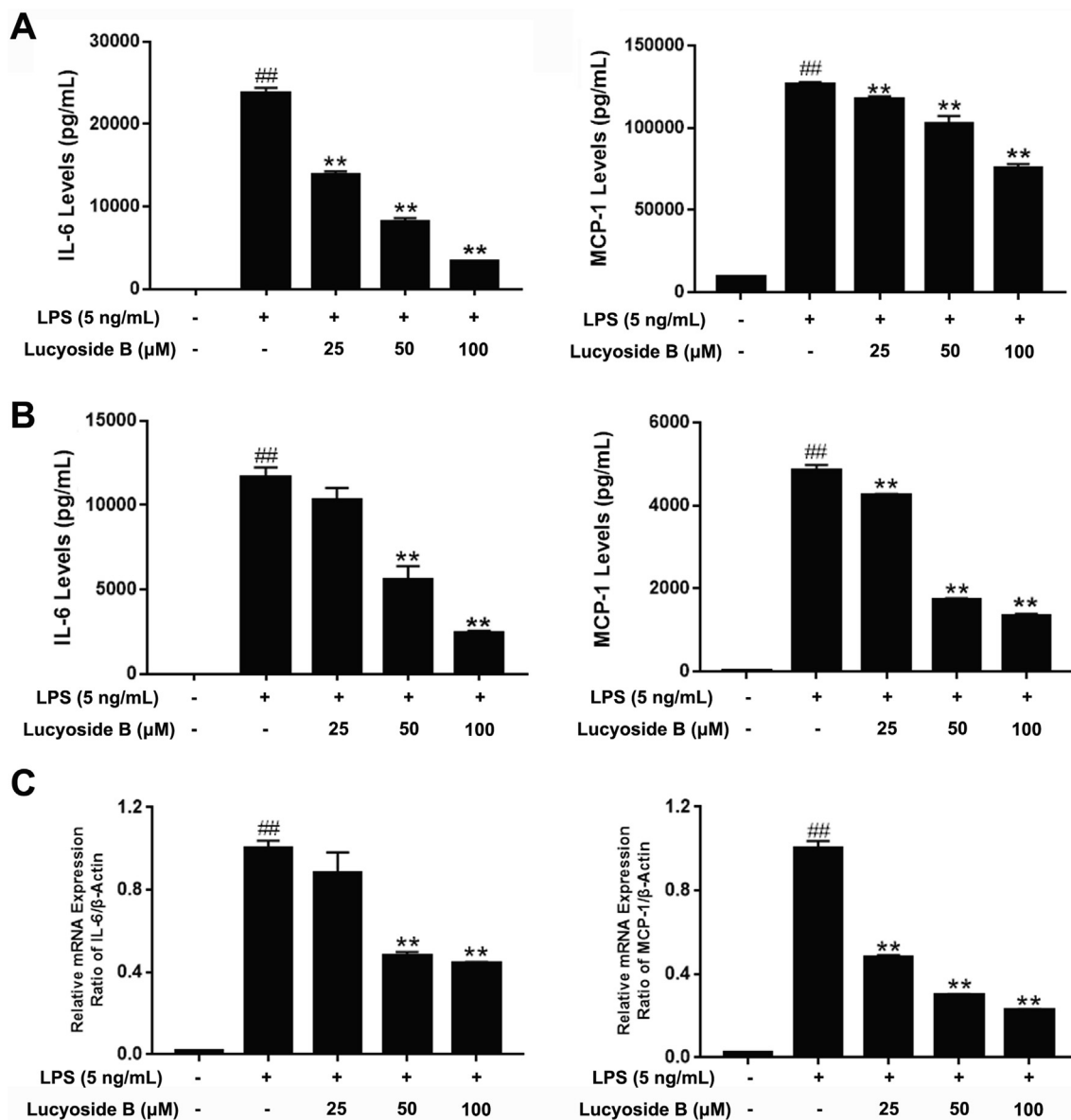

Fig. 4. Effects of lucyoside B on IL-6 and MCP-1 at the transcriptional and translational levels in LPS-primed macrophages. (A-B) Effects of lucyoside B on LPS-induced IL-6 and MCP-1 production in RAW264.7 macrophages (A) or BMDMs (B) (n = 3). The cells were pretreated with lucyoside B at the indicated concentrations for 2 h and then were exposed to LPS (5 ng/mL) for 24 h. The levels of IL-6 and MCP-1 in the supernatant were determined by ELISA. (C) Effects of lucyoside B on IL-6 and MCP-1 mRNA expression in LPS-primed RAW264.7 cells (n = 3). The cells were pretreated with lucyoside B for 2 h and then exposed to LPS (5 ng/mL) for 4 h. The mRNA expression of IL-6 and MCP-1 was determined by qPCR analysis. $^{\#\#}P < 0.01$ compared with the normal control group; $^{**}P < 0.01$ compared with the LPS-treated model group.
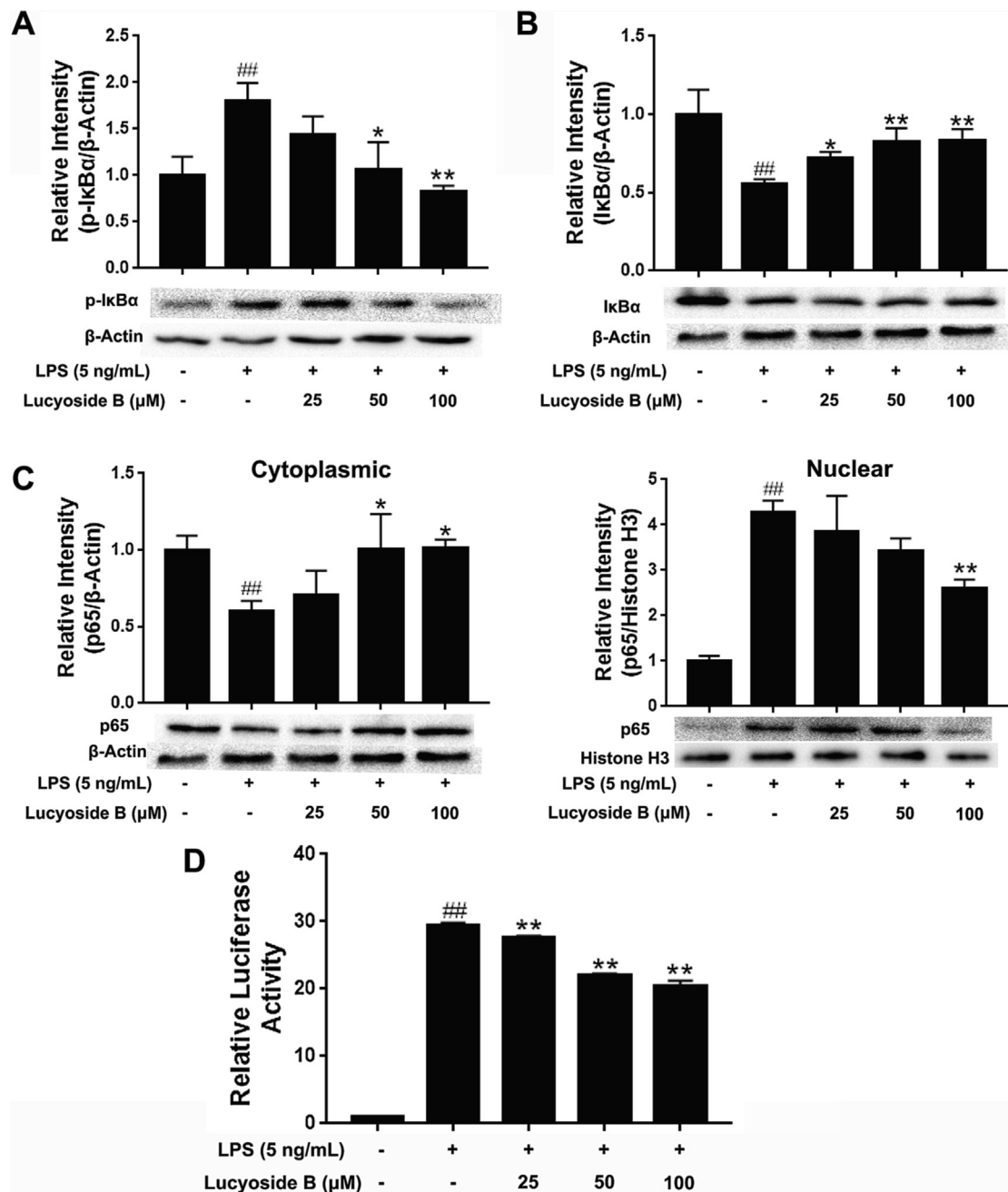
Fig. 5. Effects of lucyoside B on LPS-activated NF-κB signal transduction pathway. Effects of lucyoside B on the phosphorylation (A) and degradation (B) of IκBα (n = 3). RAW264.7 cells were pretreated with lucyoside B at the indicated concentrations for 2 h and then were exposed to LPS (5 ng/mL) for 30 min. Total protein was extracted for western blot analysis. (C) Effect of lucyoside B on the nuclear translocation of NF-κB p65 in LPS-primed RAW264.7 macrophages (n = 3). The cells were pretreated with lucyoside B at the different concentrations for 2 h and then exposed to LPS (5 ng/mL) for 30 min. Cytoplasmic and nuclear proteins were extracted for western blot analysis. (D) Effects of lucyoside B on the transcriptional activity of NF-κB (n = 3). RAW264.7 macrophages transfected with pNF-κB-TA-luc luciferase reporter plasmid were pretreated with lucyoside B at the indicated concentrations for 2 h and then were exposed to LPS (5 ng/mL) for 4 h. NF-κB activities were detected using the Luciferase Assay System. $^{\#\#}P < 0.01$ compared with the normal control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the LPS-treated model group.

lucyoside B concentration-dependently suppressed the production of IL-6 and MCP-1 in both LPS-primed RAW264.7 macrophages and BMDMs. We next investigated the effect of lucyoside B on the mRNA levels of IL-6 and MCP-1 using qPCR. Consistently, lucyoside B suppressed IL-6 and MCP-1 transcription concentration-dependently in LPS-activated RAW264.7 macrophages (Fig. 3C).

3.4. Lucyoside B suppresses LPS-induced activation of NF-κB signal transduction pathway

On the membrane of macrophages, LPS ligates TLR4 to recruit the cytoplasmic adaptor protein myeloid differentiation factor 88 to the membrane and allows for subsequent activation of the inflammatory cascades inside the cells, among which NF-κB signaling is considered paramount. To investigate whether the inhibitory effects of lucyoside B on pro-inflammatory cytokines were mediated by suppressing the activation of the NF-κB pathway, we first examined phosphorylation and
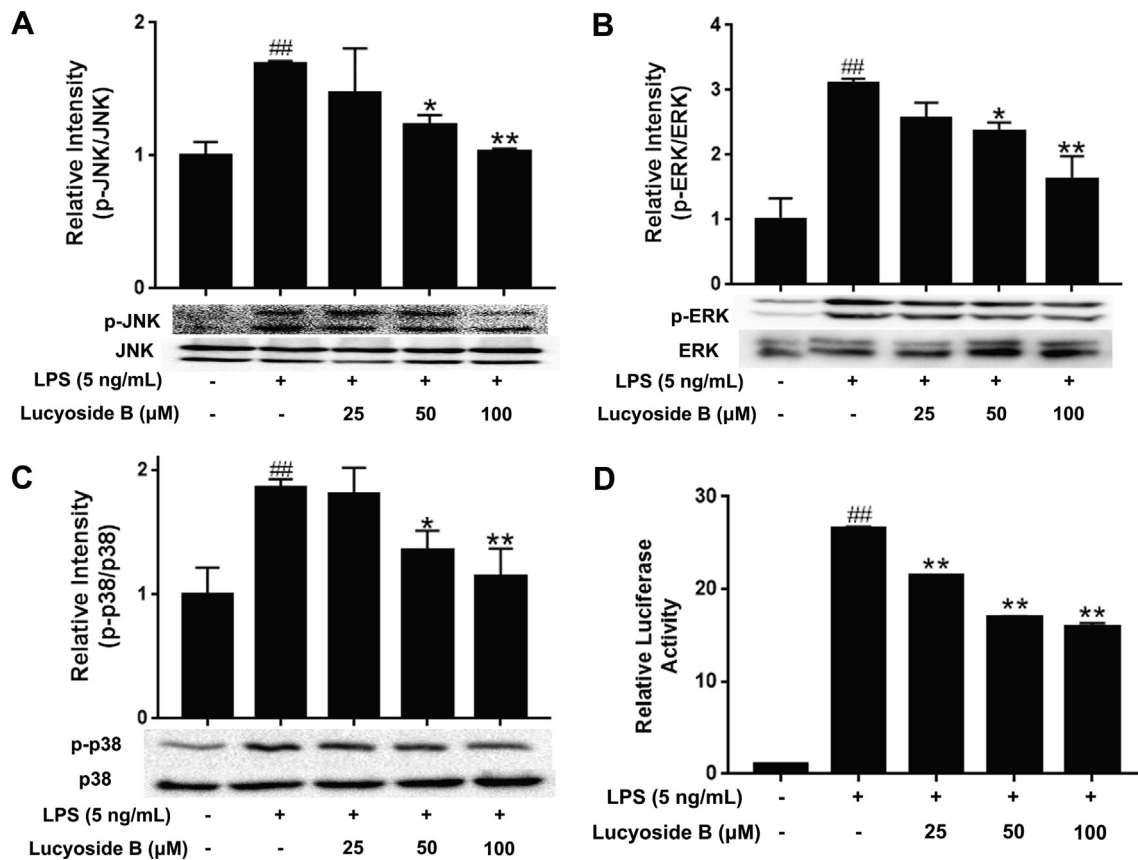
Fig. 6. Effects of lucyoside B on LPS-activated AP-1 signal transduction pathway. (A-C) Effects of lucyoside B on LPS-induced phosphorylation of JNK1/2 (A), ERK1/2 (B) and p38 (C) (n = 3). RAW264.7 cells were pretreated with lucyoside B at the indicated concentrations for 2 h and then were exposed to LPS (5 ng/mL) for 15 min. Total proteins were extracted for western blot analysis. (D) Effect of lucyoside B on the transcriptional activity of AP-1 (n = 3). RAW264.7 cells transfected with pAP-1-TA-luc luciferase reporter plasmid were pretreated with lucyoside B at the indicated concentrations for 2 h and exposed to LPS (5 ng/mL) for another 4 h. AP-1 activities were detected using the Luciferase Assay System. $^{\#\#}P < 0.01$ compared with the normal control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the LPS-treated model group.

degradation of IκBα. The obtained results showed that lucyoside B could concentration-dependently suppress the phosphorylation (Fig. 5A) and degradation (Fig. 5B) of IκBα. Then, the effect of lucyoside B on the nuclear translocation of NF-κB p65 was investigated. As shown in Fig. 5C, LPS significantly induced the translocation of p65, which was inhibited by lucyoside B. Furthermore, the luciferase assay also revealed that lucyoside B significantly suppressed the activation of NF-κB responsive elements (Fig. 5D).

3.5. Lucyoside B suppresses LPS-induced activation of AP-1 signal transduction pathway

Apart from NF-κB signal transduction pathway, the transcription of pro-inflammatory mediators is also regulated by AP-1 signaling pathway. Therefore, we next examined the effects of lucyoside B on the phosphorylation of MAPKs, which act upstream of AP-1, including JNK1/2, ERK1/2 and p38. The results indicated that lucyoside B suppressed LPS-induced JNK1/2, ERK1/2 and p38 phosphorylation in a concentration-dependent manner (Fig. 6A–C). Next, we detected the action of lucyoside B on AP-1 transcriptional activity. And the result showed that lucyoside B could also inhibit the luciferase activity in a concentration-dependent fashion (Fig. 6D).

3.6. Lucyoside B inhibits supernatant NO production in LTA-activated macrophages

To facilitate the progress of mechanism study, LTA, a TLR2 agonist, was used to stimulate RAW264.7 macrophages. The obtained result
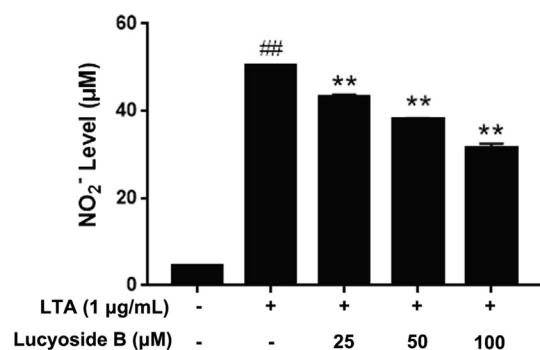

Fig. 7. Effect of lucyoside B on NO production in LTA-activated RAW264.7 cells (n = 3). The macrophages were pretreated with different concentrations of lucyoside B for 2 h and then exposed to LTA (1 μg/mL) for 24 h. Nitrite levels in the culture medium were measured by the Griess reaction. $^{\#\#}P < 0.01$ compared with the normal control group; $^{**}P < 0.01$ compared with the LTA-treated group.

indicated that lucyoside B could markedly reduce LTA-induced NO production under the same concentrations previously used (Fig. 7). Together with the fact that membrane receptor difference is the only distinction between LTA- and LPS-triggered cascades for NO production in macrophages (Chang et al., 2006), we inferred that the effect target of lucyoside B was in the cytoplasm, rather than on the membrane.

4. Discussion

Lucyosides are a series of compounds with triterpenoid saponin basic structure. Until now, 18 lucyosides (lucyoside A → R) have been isolated and identified (Du & Li, 2005; Qu et al., 2017). Triterpenoid saponins are known to be a class of compounds with anti-inflammatory property (Seki, Tamura, & Muranaka, 2015). Previous studies showed that lucyosides (total saponins) accelerated the growth of premature mice, enhanced phagocytosis of peritoneal macrophages, promoted hemolysin formation, and had the protective effect against disadvantageous stimulation, such as fatigue, nuclear radiation damage, anoxia and high temperature, which is similar to the "adaptogen" effect of ginseng (He et al., 1997). In this study, we found that lucyoside B exerted an anti-inflammatory activity in LPS-activated macrophages through inactivating nuclear transcription factors NF-κB and AP-1 thus inhibiting proinflammatory mediators (e.g. NO, IL-6 and MCP-1) release.

NO, a small diffusible molecular produced by iNOS in activated macrophages, is closely related to many inflammatory diseases (Uehara, Shida, & de Brito, 2015). We found that lucyoside B could potently decrease LPS-induced NO production in both RAW264.7 macrophages and primary macrophages (Fig. 2A and B) without influencing iNOS activity at the current tested concentrations (Fig. 2C). Subsequent research showed that the inhibitory effect of lucyoside B on iNOS was mainly attributed to the suppression of iNOS mRNA (Fig. 3A) and protein levels (Fig. 3B).

Apart from NO, macrophages also produce pro-inflammatory cytokines such as IL-6 and MCP-1 under aggression challenge. Excessive production of these cytokines leads to capillary leakage, vascular hemorrhage, tissue destruction and subsequent lethal organ failure (Scheibel et al., 2010). Thus, the expression of pro-inflammatory cytokines must be tightly controlled during the inflammatory response. Our results showed that lucyoside B decreased LPS-induced IL-6 and MCP-1 at the transcriptional (Fig. 4C) and translational (Fig. 4A and B) levels, indicating lucyoside B had the anti-inflammatory potency.

NF-κB is a pivotal regulator of inflammation and immunity that control the expression of many immunoregulatory genes (Doyle & O'Neill, 2006). In resting cells, NF-κB is sequestered in the cytoplasm by its inhibitory protein IκBα. Following LPS exposure, IκBα is phosphorylated and then degraded, the liberated p65 translocates into the nucleus to enhance the transcription of inflammatory cytokines (DiDonato et al., 1996). According to our results, lucyoside B inhibited the NF-κB transcriptional activity (Fig. 5D), which was correlated with the decreased IκBα phosphorylation (Fig. 5A), degradation (Fig. 5B) and the p65 translocation (Fig. 5C).

Upon LPS stimulation, another transcriptional factor AP-1, which is activated by MAPK family members such as JNK1/2, ERK1/2 and p38, could bind to the promoter region of various genes which govern the expressions of inflammatory cytokines. Thus, components of AP-1 signaling are recognized as important regulators in inflammatory diseases. Lucyoside B was found to inhibit phosphorylation levels of JNK1/2, ERK1/2 and p38 induced by LPS (Fig. 6A–C), which was consistent with the AP-1 suppressing by lucyoside B in the reporter gene assay (Fig. 6D).

Given the fact that lucyoside B could suppress both NF-κB and AP-1 signaling pathways, mechanism study is easy to focus on TLR4, including blocking the binding of LPS to TLR4 (Park et al., 2009), or interfering its dimerization (Miyake, 2006). To conveniently test this conjecture, LTA, a TLR2 agonist, which could also activate NF-κB and AP-1 pathways (Finney, Leaver, Evans, & Burke-Gaffney, 2012), was used for stimulating same cells. However, the obtained result showed that under the same concentrations, lucyoside B could also decrease supernatant NO production in LTA-induced macrophages (Fig. 7). Therefore, we ruled out the possibility that lucyoside B acted on TLR4 to exert anti-inflammatory activity.

5. Conclusion

Taken together, this study is the first report on the anti-inflammatory character of lucyoside B in LPS-primed macrophages. By inhibiting NF-κB and AP-1 signaling pathways, lucyoside B restrains the transcription and translation of iNOS, IL-6 and MCP-1 to exert anti-inflammatory actions. As the extracts of *Luffa cylindrica* have been reported to exert anti-inflammatory effect, our study suggests that lucyoside B is one of the effective anti-inflammatory constituents in *Luffa cylindrica*, which may be a beneficial foodstuff for inflammatory diseases.

6. Ethics statement

All animal care and experimental protocols and procedures were approved by the Committee for Care and Welfare of Laboratory Animals in Institute of Medicinal Plant Development of Chinese Academy of Medical Sciences & Peking Union Medical College. Animal studies are reported in compliance with the ARRIVE guidelines.

CRediT authorship contribution statement

Yixin Han: Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Xiaoyu Zhang:** Validation, Formal analysis, Writing - review & editing. **Ruijuan Qi:** Validation, Writing - review & editing. **Ximeng Li:** Validation, Writing - review & editing. **Yuan Gao:** Resources, Writing - review & editing. **Zhongmei Zou:** Project administration. **Runlan Cai:** Resources, Supervision. **Yun Qi:** Conceptualization, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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