Original Article

Lupalbigenin Inhibiting NF-κB Translocation Associated with Anti-Inflammatory Responses in Lipopolysaccharide Stimulated RAW 264.7 Macrophages

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Background: The dried stem of *Derris scandens* Benth. is well known as an Asian medicinal plant and is used for a variety of ailments. It is claimed to used for the relief of muscle aches and pain and revealed the presence of compounds that appear to modify inflammatory processes. Chemical analysis of *D. scandens* have revealed the presence of numerous isoflavone derivatives. Lupalbigenin, prenylated isoflavone is a key component of *D. scandens* stem ethanolic extract. Its anti-inflammatory activity in cell culture had never previously been reported which is why this study was performed.

Objective: To investigate the inflammatory activity through molecular signaling pathways of lupalbigenin from *D. scandens* aqueous ethanol extract using LPS-induced cell changed in Raw 264.7 macrophages cells.

Materials and Methods: Lupalbigenin was purified from aqueous extract of dried stem of *D. scandens*. Cell viability of lupalbigenin in Raw 264.7 cell was determined by MTT assay. Griess reagent has been used for nitric oxide determination. The protein expression of iNOS, cyclooxygenase-2 (COX-2), TNF-α, NF-κB and MAPK were performed by Western blotting. In addition, we also used immunofluorescent assay to examine the NF-κB translocation from nucleus to cytoplasm.

Results: Lupalbigenin at 1.25 and 2.5 mM effectively inhibited the LPS-induced tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) as well as nuclear factor kappa B (NF-κB). In addition, lupalbigenin prevented LPS-induced inflammation by decreasing p38 and JNK expression in mitogen-activated protein kinase (MAPK) pathway.

Conclusion: Lupalbigenin at low concentrations showed down-regulation of inflammatory gene and protein expressions as well as inhibiting NF-κB translocation. Lupalbigenin could be used as an anti-inflammatory agent.

Keywords: Lupalbigenin; Anti-inflammatory activity; Nitric oxide (NO); NF-κB translocation

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Inflammation is a natural defense response when the body is invaded by bacteria, viruses, and fungi[1]. Activation of macrophages has been detected in inflammatory tissue and induced after exposure to stimuli agents such as tumor necrosis factor-α, interferon-γ, and a microbial lipopolysaccharide (LPS)[2]. LPS is a common endotoxin derived from the outer membrane of gram-negative bacteria and stimulate series of inflammatory reactions[3]. Nuclear factor-kappa B (NF-κB) is a crucial pathway regulating transcription of inflammatory cytokines and mediators during inflammatory process activated by LPS stimulation. Therefore, molecules or chemical targeting of NF-κB signaling pathways are considered to be a potential anti-inflammatory agent[4].

Lupalbigenin, a prenylated isoflavone, is found in the stem of *Derris scandens* Benth (family: Leguminosae), the local name "Tao-Wan-Priang". It showed the most potent radical scavenger activity by thiobarbituric acid (TBA) and also a strong activity against thromboxane and leukotriene which are inflammatory cytokines in rat polymorphonuclear leukocytes (PMNs)[5]. It has been reported as an anti-cancer agent via cell cycle arrest which could induce apoptosis through the mitochondria signaling pathway in human breast cancer cell[6]. In lung cancer cells, lupalbigenin showed significant down-regulation of anti-apoptotic protein and survival protein expression that can inhibit tumor growth as well as survival in anchorage-independent conditions[7]. Recently, a novel mechanism of topoisomerase poison activity using yeast model demonstrated that isoflavone has the potential to be an anti-cancer agent[8].

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Although lupalbigenin has been shown to have many potential benefits, this study has focused on its anti-inflammatory properties, specifically the effects of lupalbigenin on NO production, iNOS, COX-2 and TNF-α protein expression in LPS-induced macrophages systematically evaluating the mechanism of action of lupalbigenin possessing anti-inflammatory activity.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY, USA) Griess reagent and iNOS antibody (ab15323) were purchased from Merck Millipore (Merck KGaA, Darmstadt, Germany) iNOS, COX-2, TNF-α, phospho-NF-κB-p65, NF-κB-p65, phospho ERK1/2, ERK1/2, phospho JNK, JNK, phospho p38, p38 and β-actin rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Specific horseradish peroxidase (HRP)-linked secondary antibody was obtained from Cell Signaling Technology, Inc. (Danver, MA, USA). All other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Preparation of Lupalbigenin

Lupalbigenin was isolated from 50% aqueous ethanol extract of stems of *D. scandens* Benth. The extract was obtained from Bangkok Lab and Cosmetic Co, Ltd. Ratchaburi province, Thailand. The crude extract (20 g) was partitioned with 5% methanol (MeOH) in dichloromethane (DCM) to afford a crude 5% MeOH/DCM extract and then subjected to vacuum-liquid chromatography on silica gel (100% DCM) to give 7 fractions (A-G). Fraction E (121 mg) was separated by normal phase column chromatography on silica gel with DCM-MeOH gradient elution. Lupalbigenin (29.5 mg) was eluted by 1% MeOH in DCM as a pale yellow solid. The structure was determined through analysis of its spectroscopic data showed ^{1}H NMR (300 MHz, $CDCl_3$+ CD_3OD) δ 7.74 (s, 1H, H-2), 7.10 to 7.15 (m, 2H, H-2′, H-6′), 6.88 (d, J = 8.2 Hz, 1H, H-5′), 6.30 (s, 1H, H-8), 5.30 (t, J = 7.1 Hz, 1H, H-2‴), 5.23 (t, J = 6.6 Hz, 1H, H-2″), 3.31 to 3.38 (m, 4H, H-1″, H-1‴), 1.78 (s, 3H, CH_3), 1.71 (s, 6H, $2CH_3$), 1.69 (s, 3H, CH_3). ESI-MS m/z: 405.8 (55%). Lupalbigenin powder was dissolved in 100% dimethyl sulfoxide (DMSO) for the stock solution and 1% DMSO as a working solution for use in cell culture experiments.

Cell culture

Macrophages cell RAW 264.7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Cell viability assay

Two sets of RAW 264.7 cells were each seeded in 96-well plates and incubated for 24 h. First set lupalbigenin at 0.625 to 20 µM was added and incubated 37°C for 24 h. The second set was pre-treated with lupalbigenin at 0.625 to 20 µM for 1 h then 1 µg/mL of LPS was added and incubated at 37°C for 24 h. After incubation, 5 mg/mL MTT solution was added to each well and incubated for 4 h in a CO_2 incubator at 37°C. Subsequently, the supernatant was removed and DMSO was added to dissolve formazan crystals. The absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek, USA).

Measurement of NO production

Cells were plated in 24-well plates and subsequently treated with 0.625 to 20 µM of lupalbigenin for 1 h then 1 µg/mL of LPS was added for 37°C for 24 h. The culture supernatant was mixed with Griess reagent (1: 1 mixture of 1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated at room temperature for 10 min. Subsequently, the NO concentration in the supernatant was determined by measuring absorbance at 540 nm (BioTek, USA).

Western blot analysis

Cells were seeded in 6-well plate and then treated with different concentrations of lupalbigenin for 1 h then stimulated with 1 µg/mL of LPS for 24 h and lysed using RIPA lysis buffer. Protein samples were electrophoresed on SDS polyacrylamide gels (8 to 12%) and the separated proteins were transferred to PVDF membranes then incubated in blocking solution (Tris-buffer/Tween 20, TBST) containing 5% BSA (w/v) for 2 h under gentle shaking at room temperature. Subsequently, the membranes were incubated with primary antibodies (iNOS, COX-2,TNF-α, phospho ERK1/2, ERK1/2, phospho JNK, JNK, phospho p38, p38 and β-actin diluted 1: 1,000 in 5% BSA in TBST) for 24 h at 4°C. After incubation, the membranes were incubated with secondary antibodies (1: 3,000) for 2 h at room temperature. Signals were developed using the ECL Western blotting detection kit and visualized on Merck Millipore (Merck KGaA, Darmstadt, Germany). The density of the band was quantified using Image J software (National Institutes of Health).

Immunofluorescence of NF-κB translocation

Cells were fixed in 4% formaldehyde for 15 min at room temperature and permeated with cold methanol for 10 min. Cells were then blocked for 1 h with 1% BSA and permeabilized by incubation with 0.4% Triton X-100 for 30 min. Subsequently, cells were washed with PBS and incubated with anti-p65 primary antibody (1: 400) at 4°C overnight. After washing with PBS, cells were incubated with a fluorescence secondary antibody (1: 200) at RT in the dark and cells were then washed with PBS. Cells were then stained with Hoechst 33342 and washed with PBS. Slides were mounted and counter-stained using vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and visualized under a laser scanning microscope (LSM 700; ZEISS, Jena, Germany).

Statistical analysis

All values are presented as mean ± standard deviation. The differences between mean values of normally distributed data were assessed by one-way analysis of variance (ANOVA) (Dundette's, Bartlett's and Sidak's multiple comparisons test) using GraphPad Prism version 8.3.4.2 (679) (GraphPad Software, San Diego, CA, USA). The criterion for differences was considered significant at $p<0.05$.

Results

Structure Identification of lupalbigenin

Structure of a pale yellow solid obtained was identified by comparison of its ^{1}H NMR data with literature reported[9]. The ESI-MS showed a molecular ion peak at m/z 405.8, which corresponded to the molecular weight of lupalbigenin (Figure 1).

Effect of lupalbigenin on RAW 264.7 macrophage viability with LPS

Cells were treated with various concentrations of lupalbigenin (0.625 to 20 µM) without and with LPS to define non cytotoxic concentration by MTT assay. Control or untreated was used 1% DMSO treated with cell and all drugs were adjusted to 1% DMSO at final solvent concentration. As a result, for lupalbigenin treated without LPS the concentration 5 µM and below has no toxicity with Raw 264.7 cells (Figure 2A). While in condition with LPS shown lupalbigenin concentration at 2.5 µM and below was not significantly affected upon a 24-h treatment whether without or with 1 mg/mL of LPS (Figure 2B).

Effect of lupalbigenin on NO production

NO is a major inflammatory mediator during the immune-inflammatory response which can be produced by

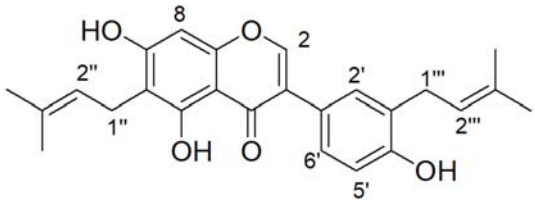

Figure 1. Lupalbigenin of structure.

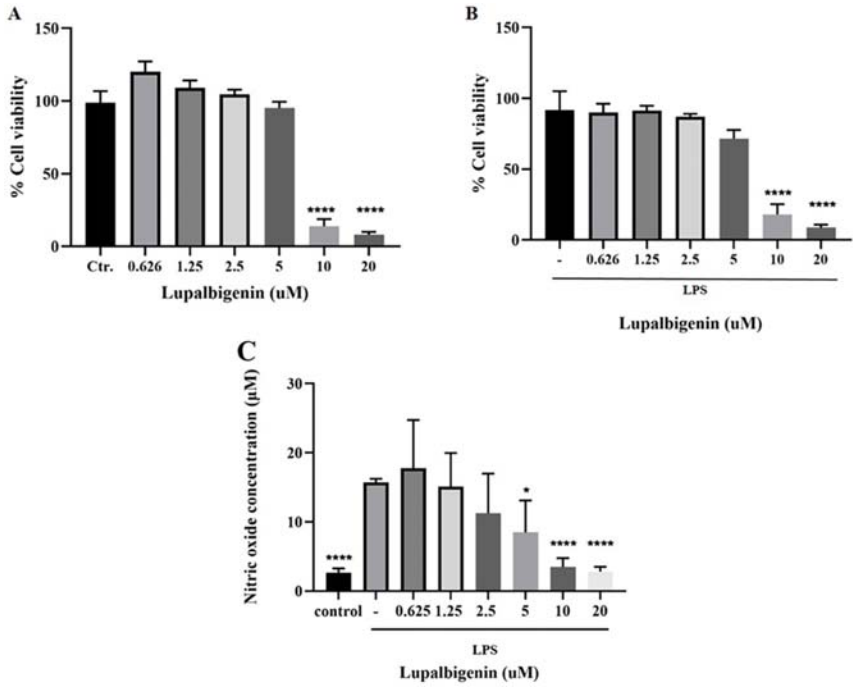

Figure 2. Cell viability of lupalbigenin on RAW 264.7 macrophage without (A) and with 1 mg/mL of LPS (B). Cells were treated with 0.625 to 20 µM lupalbigenin and detected by MTT assay. Effect of lupalbigenin on NO production in LPS induced RAW 264.7 macrophage cells. After 24 h treatment with 0.625 to 20 µM lupalbigenin, NO production was measured by the Griess reaction assay (C). Data are expressed as mean ± standard deviation (n=6). This figure has been generated using GraphPad, the following annotations relate to the p-value. **** denotes a p-value <0.0001, *** denotes a p-value = 0.0001 to 0.001, ** denotes a p-value = 0.001 to 0.01, * denotes a p-value = 0.01 to 0.05 and no annotation denotes a p-value ≥0.05.

macrophages cells. In this experiment Greiss reagent assay has been used for NO determination with LPS-induced in RAW 264.7 macrophages cell. The result showed 1 ug/mL of LPS can be significantly induced NO production compare with control (untreated cell). The concentration of 2.5 to 20 µM of lupalbigenin inhibited nitric oxide production in LPS-induced cells. However, 5, 10 and 20 µM lupalbigenin has cytotoxicity in RAW 264.7 macrophages with LPS-induced, Therefore lupalbigenin at concentrations of 1.25 and 2.5 µM were chosen for further study (Figure 2C).

Inhibition of iNOS, COX-2 and TNF-α protein expression by western blotting

Inflammatory protein expression upon 1.25 and 2.5 µM lupalbigenin treatment was determined by Western blot analysis. At 1.25 and 2.5 µM lupalbigenin treated LPS-stimulated RAW 264.7 cells showed inhibition of iNOS protein expression and significantly decreased both COX-2 and TNF-α level as compared with the LPS treated RAW 264.7 cells (Figure 3).

Detection of NF-κB translocation

The effect of lupalbigenin on NF-κB translocation was determined using immunofluorescence. The result showed that lupalbigenin inhibited p-NF-κB translocation from cytoplasm to nucleus. These results reveal that lupalbigenin regulates the inflammatory response of RAW 264.7 cells by inhibiting the translocation of NF-κB p65 (Figure 4).

Lupalbigenin inhibited P38 phosphorylation on MAKP pathway

As MAPK protein is crucial for nitric oxide and pro-inflammatory cytokine production, therefore it is a potential efficient target of lupalbigenin. The results showed that 1.25 µM Lupalbigenin increased phosphorylated ERK1/2 while decreased JNK phosphorylation. However, p38 phosphorylation was markedly decreased upon treated with 1.25 µM and 2.5 µM lupalbigenin (Figure 5).

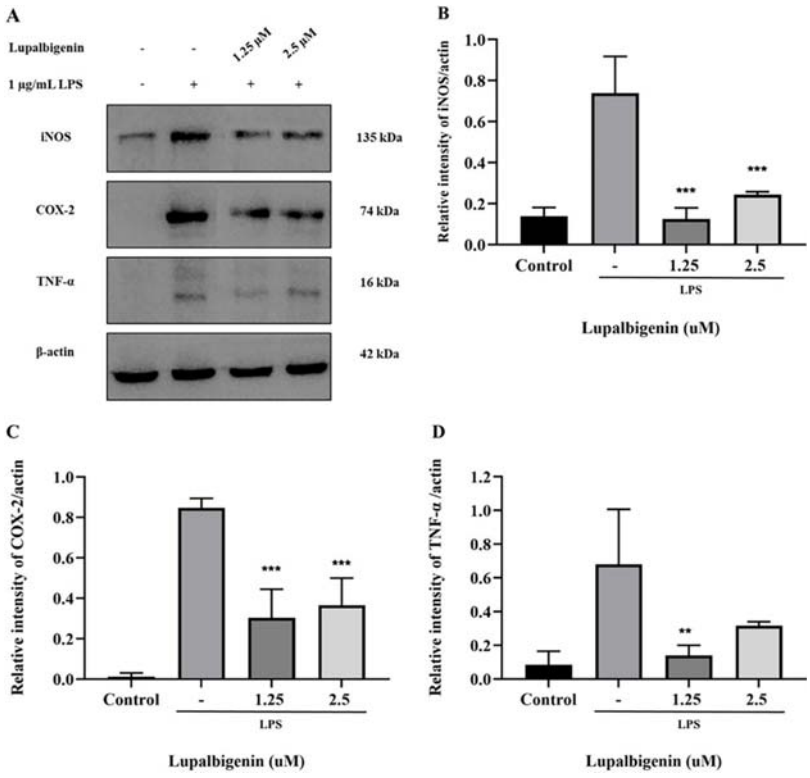

Figure 3. Effect of lupalbigenin on iNOS, COX-2 and TNF-α protein expression. Cells were treated with 1.25 and 2.5 µM lupalbigenin for 1 h and then treated with LPS for 24 h vs. the LPS-induced only group. Total protein was analyzed by Western blot analysis (A). The relative intensity of iNOS (B), COX-2 (C) and TNF-α (D) protein expression were normalized by β-actin and bars indicate mean ± standard error of three independent experiments. This figure has been generated using GraphPad, the following annotations relate to the p-value. **** denotes a p-value <0.0001, *** denotes a p-value = 0.0001 to 0.001, ** denotes a p-value = 0.001 to 0.01, * denotes a p-value = 0.01 to 0.05 and no annotation denotes a p-value ≥0.05.

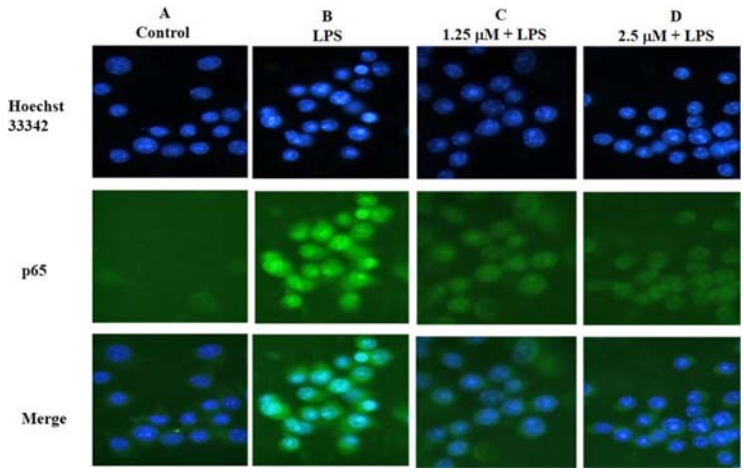

Figure 4. Lupalbigenin effectively inhibited NF-κB p65 translocation from cytoplasm to nucleus. Raw cell without LPS as control (A), Raw 264.7 cells with 1 mg/mL of LPS only (B), pretreated 1 h with 1.25 µM lupalbigenin (C) and 2.5 µM lupalbigenin (D). Incondition B, C and D were all subsequently induced with 1 mg/mL of LPS for 3 h. NF-κB, p65 localization was visualized (green color) and Hoechst 33342 was used for nuclear staining (blue color) by fluorescence confocal microscopy (200x).

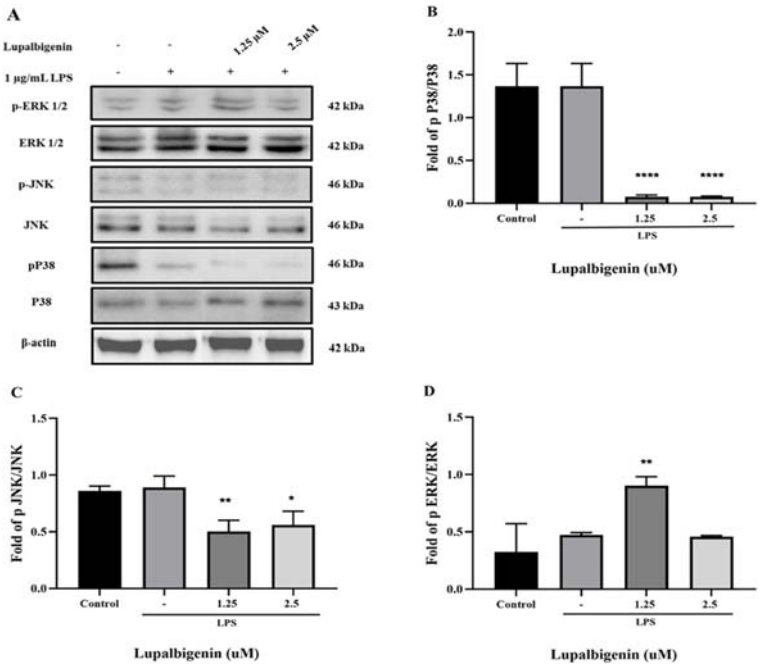

Figure 5. Effect of lupalbigenin on LPS-induced phosphorylation of MAPKs pathway in RAW 264.7 cells. Expression of phosphorylated and non-phosphorylated form of p38, JNK and ERK1/2 were detected by Western blot analysis (A). Relative protein levels were quantified by image J and normalized to β-actin level (B), (C) and (D), respectively. Bars indicate mean ± standard deviation of three independent experiments. This figure has been generated using GraphPad, the following annotations relate to the p-value. **** denotes a p-value <0.0001, *** denotes a p-value = 0.0001 to 0.001, ** denotes a p-value = 0.001 to 0.01, * denotes a p-value = 0.01 to 0.05 and no annotation denotes a p-value ≥0.05.

Discussion

Previous research reported that lupalbigenin from *D. scandens* extract inhibited eicosanoid synthesis including COXs and 5-loxs with IC_{50} of 3 μM and 6 μM and the results from lactate dehydrogenase (LDH) assay showed that 125 μg/mL lupalbigenin was toxic to leucocyte cells[10]. Houng Le Tuan Anh et al, 2017 also reported 30 μM lupalbigenin from *Cudrania tricuspidata* induced cell death in LPS-stimulated RAW 264.7 cell[11]. In our results, MTT assay was used to define nontoxic concentration of lupalbigenin with and without 1 mg/mL of LPS and found that at 5, 10 and 20 μM lupalbigenin with LPS-induced were toxic to RAW 264.7 cell (Figure 2B). As nitric oxide determination using Griess reagent is a well-known anti-inflammatory screening process, therefore, NO inhibition was determined. The results indicated that 2.5 to 20 μM lupalbigenin could inhibit NO production as compared with the LPS treated only (Figure 2C) however, the concentration from 5 μM and above are toxic with Raw cell therefore lupalbigenin at 1.25 μM, 2.5 μM have been chosen to do further experiments. As previously reported lupalbigenin could inhibit COXs and 5-loxs by ELISA our result found lupalbigenin at 1.25 μM and 2.5 μM showed significantly decreased COX-2, and TNF-α protein expression by Western blotting (Figure 3C and 3D). Our results implied that the noncytotoxic concentrations at 1.25 μM and 2.5 μM lupalbigenin still showed significant negative regulation of inflammatory protein expression in RAW 264.7 cell. Nuclear factor-Kappa B (NF-κB) a crucial pathway to regulate the transcription of inflammatory cytokines and mediators during inflammatory process activated by LPS stimulation was determined[12]. We performed immunofluorescence assay to investigate the translocation of NF-κB from cytoplasm to nucleus. As a result, the LPS-stimulated cells showed high capacity of NF-κB translocation while 1.25 μM and 2.5 μM lupalbigenin significantly decreased translocation (Figure 4C and 4D) which eventually could not continue the process of inflammatory gene transcription and protein translation. Furthermore, the effect of lupalbigenin on mitogen-activated protein kinase (MAPK) pathway was determined. MAPK containing three sequentially activated protein kinases, are key components of a series of vital signal transduction pathways that regulate processes such as cell proliferation, cell differentiation, and cell death in eukaryotes from yeast to humans)[13]. MAPK regulates inflammatory response via p38 involved in regulating the expression of iNOS and TNF-α gene in the macrophages whereas ERK and JNK are involved in regulations of pro-inflammatory cytokine and iNOS[14,15]. Our results showed at low concentration lupalbigenin inhibited the LPS-induced phosphorylation of JNK and significantly inhibited phosphorylation of p38. These results suggest that lupalbigenin could be effective for protecting LPS induced cell death. The cell signaling pathway and in vivo study should be performed further.

Conclusion

The result from incubated 1.25 μM and 2.5 μM lupalbigenin with 1 μg/mL LPS could be a strong drug to control the down-regulation of COX-2, iNOS, and TNF-α protein expression. Lupalbigenin has potential to inhibit the upstream of inflammatory activity control via NF-κB translocation and MAPKs. It down regulated the phosphorylation of p38 and JNK in LPS-induced RAW 264.7 macrophages cells. In summary, low concentrations of lupalbigenin could be used as the ant-inflammatory agent. Further molecular signaling pathways and in vivo study should be performed.

What is already known on this topics?

Lupalbigenin is a prenylated isoflavone which can be purified from dried stem of D. scandens and showed potent inhibition for anti-oxidant activity, anti-cancer and inhibit thromboxane and polymorphonuclear leukocytes (PMNs).

What this study adds?

In this study, we investigated the mechanism of lupalbigenin on inflammatory response in lipopolysaccharide (LPS)-treated RAW 264.7 macrophages. Our results demonstrated that lupalbigenin effectively inhibited LPS-induced tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) as well as nuclear factor kappa B (NF-κB). Our results suggest that lupalbigenin may attenuate the inflammatory response via inhibition of NF-κB activation associated with down regulation of MAPK expression in LPS-induced RAW 264.7 macrophages.

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Potential conflicts of interest

The authors declare no conflict of interest.

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