S-deoxydihydroglyparvin from *Glycosmis parva* inhibits lipopolysaccharide induced murine macrophage activation through inactivating p38 mitogen activated protein kinase

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ABSTRACT

Macrophages play major roles to produce several pro-inflammatory and inflammatory mediators in chronic inflammatory diseases. All current anti-inflammatory drugs target these mediators to alleviate inflammation. Searching for new anti-inflammatory agents is always needed due to problems from the clinical use of current anti-inflammatory drugs. We intended to evaluate the anti-inflammatory potential of three main compounds, arborinine, methylatalaphylline, and *S*-deoxydihydroglyparvin (DDGP), from *Glycosmis parva* leaves and branches on macrophage stimulated by lipopolysaccharide (LPS). Only DDGP demonstrated a potent inhibitor of LPS-activated macrophages. Results indicated that the mRNA level of inducible nitric oxide synthase (iNOS) was inhibited by the treatment in accompany with the decreased nitric oxide (IC50 at $3.47 \pm 0.1\ \mu M$). DDGP was shown to suppress tumor necrosis factor-α, interleukin (IL)-1, and IL-6 at the mRNA expression and at the released protein levels. In addition, DDGP inhibited the several chemokines, monocyte chemoattractant protein-1 and macrophage inflammatory proteins-1α, and enzymes for prostaglandin (PG) synthesis. It also inhibited PGE2 production. On LPS signaling pathways, DDGP profoundly decreased phosphorylation of p38 mitogen-activated protein kinase (MAPK) in the LPS-treated cells. It had little or no effect on the activation of JNK, ERK and nuclear factor kappa B. In conclusion, results suggested that DDGP from *G. parva* inhibited expression and production of inflammatory molecules in LPS-activated macrophages through suppressing p38 MAPK activation. DDGP should be a good candidate anti-inflammatory agent in the future.

Key words: Anti-inflammation, *Glycosmis parva*, lipopolysaccharide, macrophage, *S*-deoxydihydroglyparvin

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INTRODUCTION

Inflammation is a defense mechanism of the body against harmful stimuli. Dysregulated inflammation is involved

in various chronic inflammatory diseases.[1] Activated macrophages are crucial cells for the pathogenesis of chronic inflammation by generating several inflammatory molecules.[2] The mitogen-activated protein kinase (MAPK) signaling pathways and activation of nuclear factor kappa B (NF-κB) are the main intracellular signaling cascades involve in the production of these inflammatory mediators.[3] Syntheses and activities of these mediators become the targets of current anti-inflammatory drugs. Side effects and high cost of these drugs present a burden for patients. Therefore, the search for novel anti-inflammatory agents is still needed.[4] Medicinal plants are rich sources for searching new anti-inflammatory agents.[5] *Glycosmis parva* Craib is a plant in the Rutaceae distributed mainly in Thailand. The ethyl acetate extracts of *G. parva* leaves and branches previously demonstrated antiviral activities against herpes simplex viruses.[6] The extract of *G. parva* leaves arrested the cell cycle and induced apoptosis of colorectal cancer HT-29 cells in part by suppressing cyclooxygenase-2 (COX-2) expression.[7] We found that the ethyl acetate extracts of *G. parva* leaves and branches potently suppressed lipopolysaccharide (LPS)-induced macrophage J774A.1 cell activation by decreasing pro-inflammatory cytokines, COX-2, and inducible nitric oxide synthase (iNOS) expressions (unpublished data). Six acridone alkaloids and four sulfur-containing propanamide derivatives were isolated from the extracts, and their structures were well elucidated.[6] A new sulfur-containing propanamide *S*-deoxydihydroglyparvin (DDGP) from the leaves, and two acridone alkaloids, arborinine (ABN) from the leaves and *N*-methylatalaphylline (MPL) from the branches, were main compounds chosen for this study. None of them have been reported for anti-inflammatory activities. We predicted those some of these compounds may have anti-inflammatory activities similar to that of the ethyl acetate extracts. Therefore, we intended to study the anti-inflammatory potential and the mechanisms of actions of the compounds using LPS-induced macrophage model.

MATERIALS AND METHODS

Dulbecco's modified eagle medium (DMEM) and medium reagents were from Gibco (USA). Dexamethasone (DEX), dimethyl sulfoxide (DMSO), LPS (*Escherichia coli* O26:B6), and resazurin were from Sigma-Aldrich (USA). Griess reagent system and ImProm-II™ reverse transcription system were from Promega Corporation (USA). Primers for real-time polymerase chain reaction (PCR) were from Bio Basic Inc. (Canada). Tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and PGE2 ELISA kits were from ImmunoTools (Germany), Thermo Fisher Scientific (USA), and R&D Systems (USA). RIPA lysis buffer was from Abcam (UK). Cell fractionation kit and all antibodies (against ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, phospho-JNK, NF-κB p65, IκB-α, proliferating cell nuclear antigen [PCNA], and GAPDH) were purchased from Cell Signaling Technology (USA). HRP substrate was from Merck Millipore (USA).

Cell culture

Mouse macrophage RAW 264.7 cells (ATCC, USA) were used in the present study. The cells were cultured in DMEM supplemented with 100 U/ml penicillin and 100 µg/mL streptomycin and 10% fetal bovine serum, in appropriate cell culture condition. The cells were prepared at 4×10^5 cells/ml in suitable well plates for 24 h and treated with 100 ng/ml of LPS.
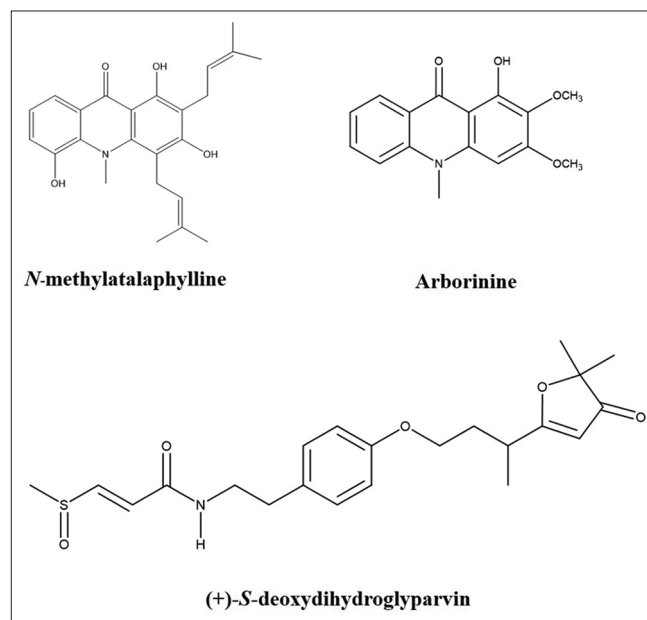
Test compounds

DDGP, ABN, and MPL were isolated from ethyl acetate extracts of *G. parva* and well characterized [Figure 1].[6] These compounds were stocked in DMSO and made to the final concentrations containing 0.2% DMSO. The positive control was 10 µM DEX.

Nitric oxide determination

LPS-activated cells were incubated with 3 and 10 µM of DDGP, ABN, and MPL for 24 h. Nitric oxide (NO) level was assessed by the Griess reaction. Concentrations of NO were derived from the standard curve of standard nitrite solutions.

For determining the IC50 value of DDGP, LPS-activated cells were treated with DDGP at 0.3125–10 µM for determining NO production as mentioned above. After assessing the IC50, DDGP at 1.25, 2.5, 5, and 10 µM was used in all experiments.

Figure 1: Structures of compounds isolated from ethyl acetate extracts of *Glycosmis parva* leaves and branches

Cell viability assay

The cytotoxic effect of the test compounds was evaluated by resazurin assay.[8] The remaining treated cells from NO determination were incubated with 50 µg/ml resazurin for 4 h at 37°C and analyzed the results of measurement at 570 nm and 600 nm.

Quantitative polymerase chain reaction

LPS-activated cells were treated with 1.25–10 µM DDGP for 4 h and/or 24 h at 37°C. Total RNA of the cells was collected and extracted using RNA isolation solution and converted to cDNA using Improm-II Reverse Transcription system. The cDNA samples were used to amplify genes of interest by real-time PCR in StepOnePlus real-time-PCR System (Thermo, USA) using qPCR green master mix and the primers of the investigated genes showed in Table 1. A loading control was β-actin. Levels of gene expression were quantified using $2^{-\Delta\Delta CT}$ method and indicated as the percentage changes from LPS-activated control.

Enzyme-linked immunosorbent assay

LPS-activated cells were incubated with 1.25–10 µM DDGP for 24 h at 37°C. The concentrations of IL-1β, IL-6, TNF-α, and PGE2 were assessed with ELISA kits followed the manufacturer manuals.

Western blot analysis

LPS-activated cells were treated with 1.25–10 µM DDGP for 30 min. Whole cell proteins were isolated using RIPA lysis buffer. Cytosolic and nuclear proteins were fractionated using cell fractionation kit. Ten micrograms protein per sample was run in 7.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked and blotted with appropriated specific primary antibodies against ERK, JNK, p38, IKB, p65 NF-κB, GAPDH, PCNA, phosphorylated ERK, phosphorylated JNK, and phosphorylated p38 (1:1000 dilution) at 4°C overnight, washed, blotted with HRP secondary antibody (1:2000 dilution) for 1 h, washed, and added HRP substrate. The density of each protein was determined using a chemiluminescence detector (C-DiGit® Blot Scanner, USA).

Statistical analysis

The data are shown as mean with the standard error of the mean of three independent experiments. Results of tested compounds were compared to the suitable control using one-way analysis of variance followed by *post hoc* test (Turkey's). IBM SPSS software version 22 (IBM Corp., USA) was utilized.

RESULTS

Effects of the compounds on nitric oxide production

We first evaluated the inhibitory potential of MPL, ABN, and DDGP on LPS-induced macrophage activation. DDGP had the most inhibitory effect on NO production [Figure 2a] without any cytotoxicity [Figure 2b]. At 10 µM, MPL, ABN, and DDGP inhibited NO production to 52%, 78%, and 19%, respectively, compared to the LPS-activated control. DDGP demonstrated a good candidate to investigate its anti-inflammatory activity.

Effect of DDGP on nitric oxide production

We identified the optimal concentrations of DDGP for evaluating its anti-inflammatory activity in detail by determining IC50 value of NO inhibition. DDGP suppressed NO production with IC50 3.47 ± 0.1 µM [Figure 3a]. DDGP at 1.25–10 µM was chosen for further evaluation. The suppressive effect of DDGP on NO generation was confirmed by determining iNOS expression, which is an inducible enzyme for NO generation. iNOS did not express in resting macrophages [Figure 3b]. DDGP decreased iNOS mRNA level in the LPS-activated cells in a similar pattern to its effect on NO production [Figure 3b].
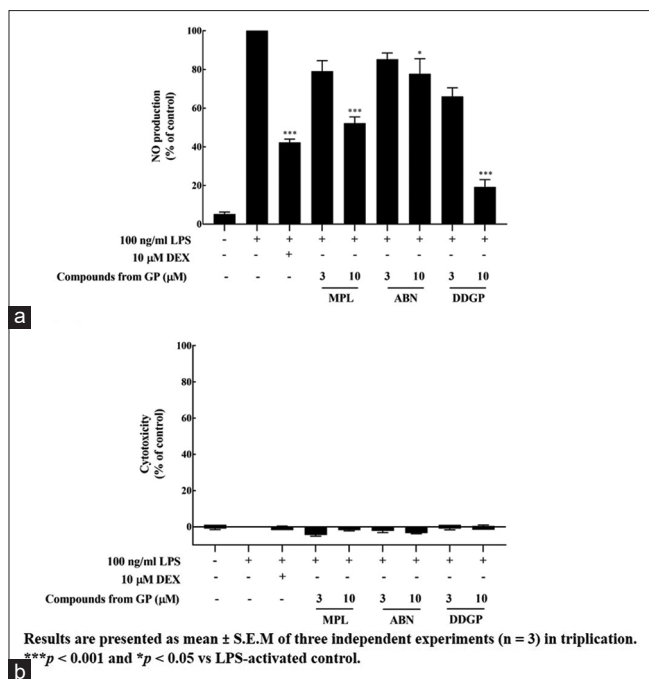
Effect of S-deoxydihydroglyparvin on the pro-inflammatory cytokines

We elucidated the possible target site of DDGP beyond iNOS/NO production by investigating its effect on pro-inflammatory cytokines. The mRNA and protein levels of key cytokines including IL-1, IL-6, and TNF-α were evaluated by real-time PCR and ELISA. DDGP downregulated the expression of these cytokines in LPS-activated cells after 4 [Figure 4.1a-c] and

Table 1: Primer sequences of the investigated genes for real-time polymerase chain reaction

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
TNF-α	TTGACCTCAGCGCTGAGTTG	CCTGTAGCCCACGTCGTAGC
IL-1β	CAGGATGAGGACATGAGCACC	CTCTGCAGACTCAAACTCCAC
IL-6	AGGACCAAGACCATCCAATTCA	GCTTAGGCATAACGCACTAGG
MCP-1	ACTGAAGCCAGCTCTCTCTTCCTC	TTCCTTCTTGGGGGTCAGCACAGAC
MIP-1α	GCCCTTGCTGTTCTTCTCTGT	GCCCTTGCTGTTCTTCTCTGT
iNOS	CCCTTCCGAAGTTTCTGGCAG	GGTGTCAGAGCCTCGTGGCT
COX-2	CACTACATCCTGACCCACTT	ATGCTCCTGCTTGAGTATGT
mPGES-1	CAGCCTAATGTTCAGCGACA	CCTTGAGCTGACAGCCTACC
B-actin	GTGGGCCGCCCTAGGCACCAG	GGAGGAAGAGGATGCGGCAGT

TNF-α: Tumor necrosis factor-α, IL: Interleukin, MCP-1: Monocyte chemoattractant protein-1, MIP-1α: Macrophage inflammatory proteins-1α, iNOS: Inducible nitric oxide synthase, COX-2: Cyclooxygenase-2

Figure 2: Effects of *N*-methylatalaphylline, arborinine, and *S*-deoxydihydroglyparvin on nitric oxide production (a) and cell viability (b) in lipopolysaccharide-activated RAW 264.7 macrophages

24 h [Figure 4.2a-c]. It also decreased protein levels of these cytokines in the supernatant [Figure 4.3 a-c]. These results imply that DDGP may act at the early step of LPS-induced macrophage activation by suppressing the protein levels of pro-inflammatory cytokines, which can induce the generation of chemokines, enzymes, and molecules involved in the inflammatory process.

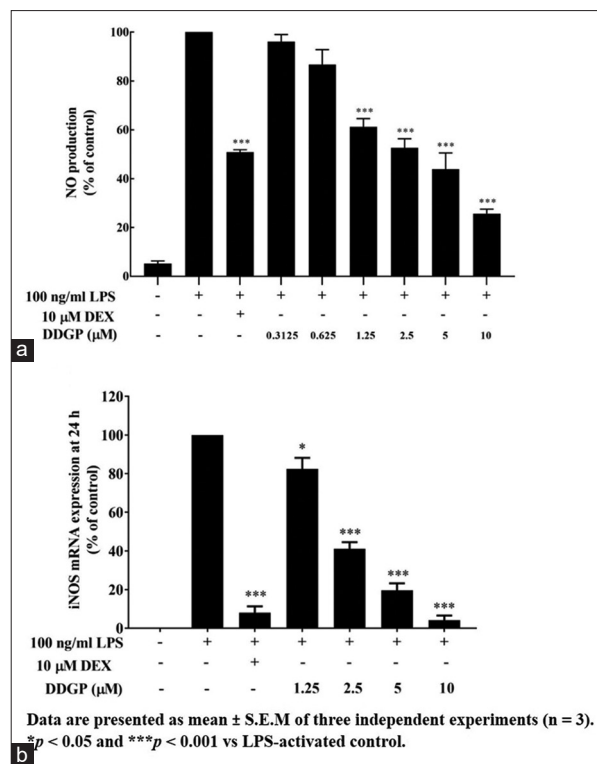### Effect of *S*-deoxydihydroglyparvin on chemokine expression

DDGP at 5 and 10 µM downregulated the mRNA expression of both monocyte chemoattractant protein-1 (MCP-1) shown in Figure 5a and macrophage inflammatory proteins-1α (MIP-1α) in Figure 5b in LPS-activated macrophages.

Effect of *S*-deoxydihydroglyparvin on cyclooxygenase-2 and mPEGS-1 expression and on PGE2 production

DDGP significantly decreased mRNA levels of inducible enzymes, COX-2 [Figure 6a], and mPGES-1 [Figure 6b]. It also decreased the production of PGE2 [Figure 6c].

Effect of *S*-deoxydihydroglyparvin on mitogen-activated protein kinase signaling molecules and nuclear factor kappa B activation

To verify the mechanism of action, the proteins in regulation of macrophage activation were further verified by western blot analysis after treating the cells with DDGP for 30 min. DDGP profoundly suppressed the phosphorylation of p38 MAPK [Figure 7c]. At 10 µM,
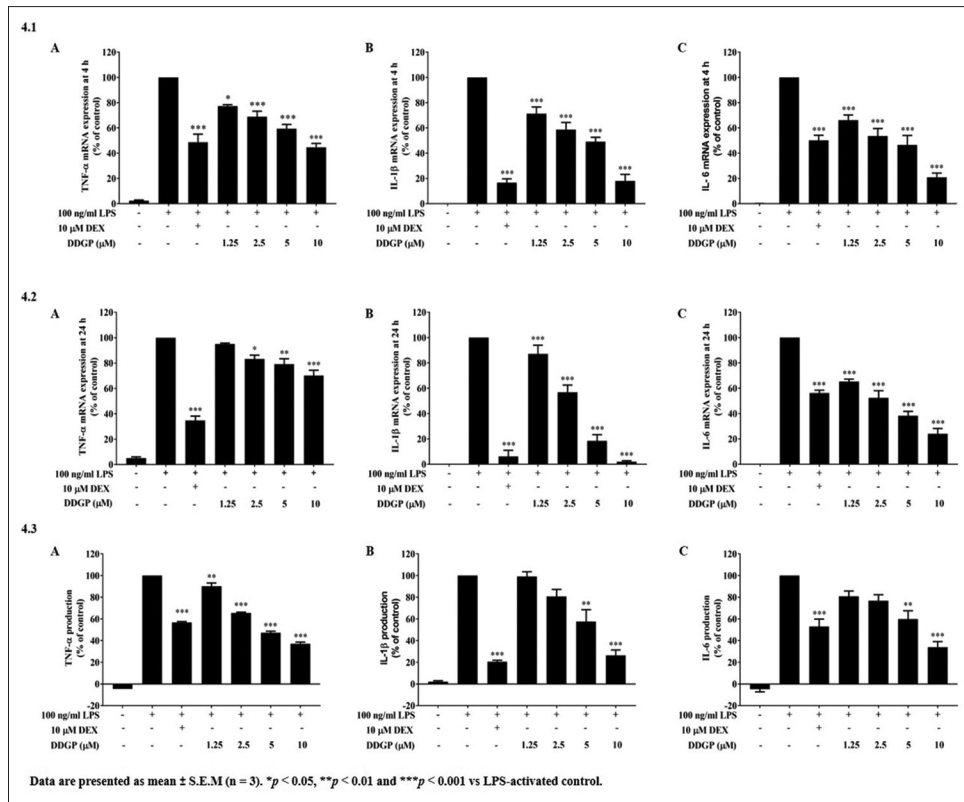

Figure 3: Effects of *S*-deoxydihydroglyparvin on nitric oxide production (a) and inducible nitric oxide synthase expression (b) in lipopolysaccharide-activated RAW 264.7 macrophages

it significantly decreased ERK phosphorylation >50% compared to the LPS control [Figure 7a]. DDGP did not have an effect on JNK phosphorylation [Figure 7b] and NF-κB activation [Figure 8]. These results propose that DDGP suppresses LPS-induced macrophage activation mainly by blocking p38 signaling pathway.

DISCUSSION

Activated macrophages and their products are key targets for developing novel anti-inflammatory agents. LPS-induced macrophage activation is a classical model used for evaluating candidate anti-inflammatory compounds. LPS activates macrophages to generate pro-inflammatory cytokines which induce the production of chemokines, adhesion molecules, inducible enzymes iNOS, and COX-2 for NO and PGE2 production.[3,9] RAW264.7 macrophages were used in the present study for evaluating anti-inflammatory effects of ABN, MPL, and DDGP.

Only DDGP demonstrated a potent inhibitor on NO production in LPS-activated macrophages. In inflammatory process, NO is mainly generated by iNOS, which expresses in macrophages at activated stage.[10] DDGP suppressed NO production through downregulating iNOS expression. It was shown that LPS and pro-inflammatory cytokines induced iNOS expression through MAPK and NF-κB activation.[9] Excessive pro-inflammatory cytokine generation was shown

Figure 4: Effects of *S*-deoxydihydroglyparvin on pro-inflammatory cytokine expression and production. The expression of tumor necrosis factor-α (A), interleukin-1β (B), and interleukin-6 (C) was determined after 4 h (4.1) and 24 h (4.2) of treatment. The cytokine levels were determined after 24 h exposure (4.3)
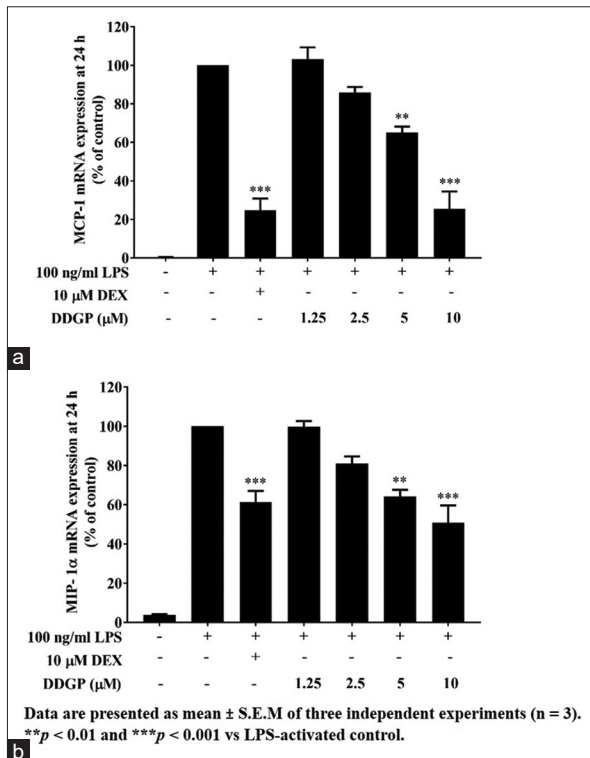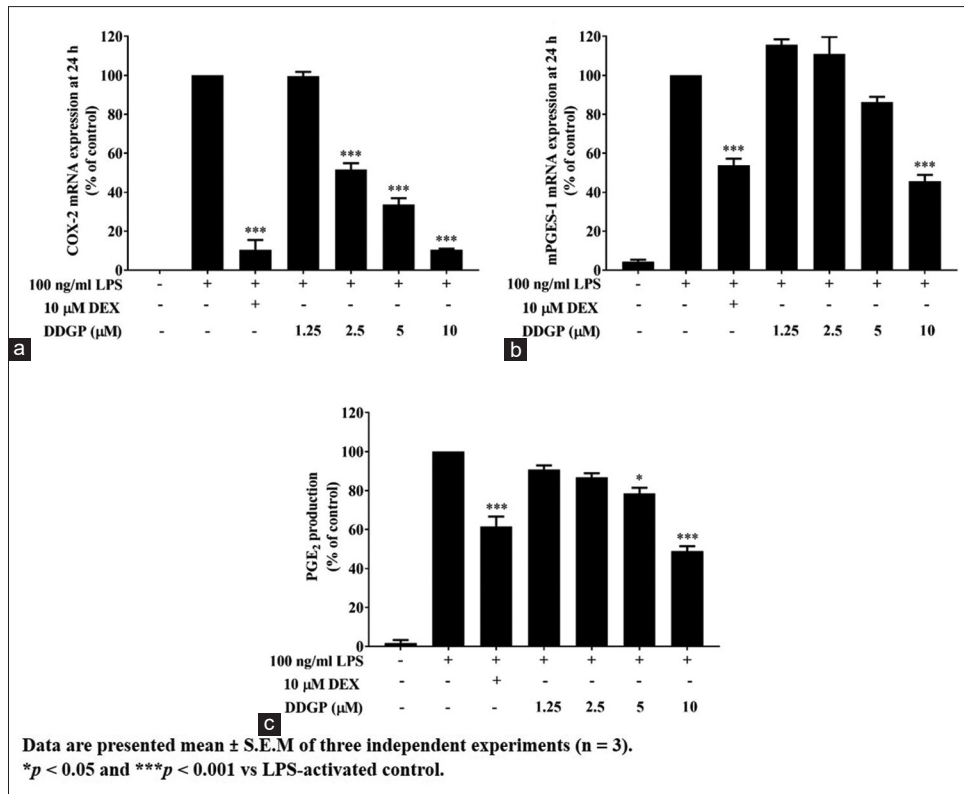

Figure 5: Effects of *S*-deoxydihydroglyparvin on the expression of chemokines, monocyte chemoattractant protein-1 (a), and macrophage inflammatory proteins-1α. (b) In lipopolysaccharide-activated RAW 264.7 macrophages

to involve in pathogenesis and progression of many inflammatory diseases.[11,12] Therefore, inhibiting the induction as well as effects of pro-inflammatory cytokine should be a potential therapeutic strategy. We found that DDGP decreased the induction of TNFα, IL-6, and IL-1β by downregulating their gene expression. Production of these cytokines is the early step of LPS-induced macrophage activation. These cytokines amplify LPS activity by upregulating the production of chemokines, iNOS/NO, and COX-2/PGE2.[2,10,13] These results suggest that DDGP inhibits in the early step of LPS-induced macrophage activation.

DDGP downregulated the expression of MCP-1 and MIP-1α, which are potent chemokines for recruiting monocytes and macrophages to inflammation areas, leading to the continuation of the inflammatory process.[2,14] DDGP also downregulated mPGES-1 and COX-2 expression leading to the decrease of PGE2 production. In activated macrophage, COX-2 catalyzes arachidonic acid to PGH2 and mPGES-1 catalyzes PGH2 to PGE2.[13] At the inflamed site, activated macrophages produce high levels of PGE2, which is a potent inflammatory mediator.[15]

LPS acts through toll-like receptor-4 to activate two main signaling pathways, MAPK signaling pathways and NF-κB activation, to induce inflammation.[3,16,17] TNF-α and IL-1 also act through these two pathways to
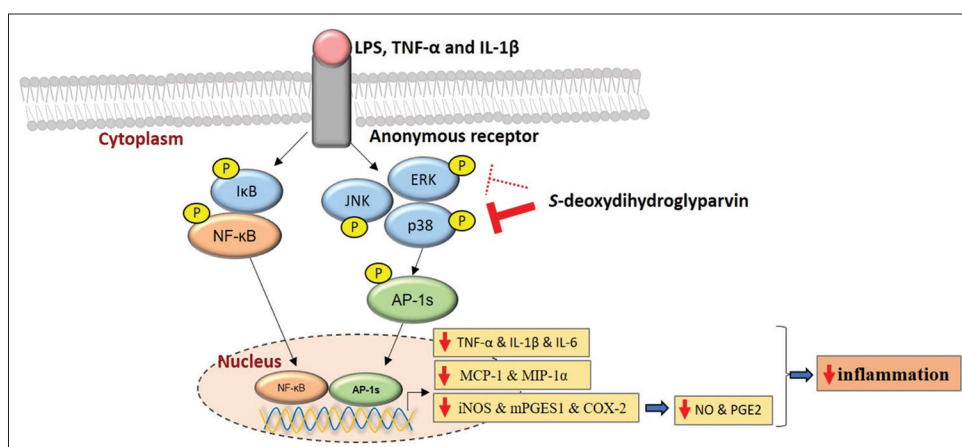
Figure 6: Effects of *S*-deoxydihydroglyparvin on the expression of cyclooxygenase-2 (a), mPGES-1 (b), and the production of PGE2 (c) in lipopolysaccharide-activated RAW 264.7 macrophages
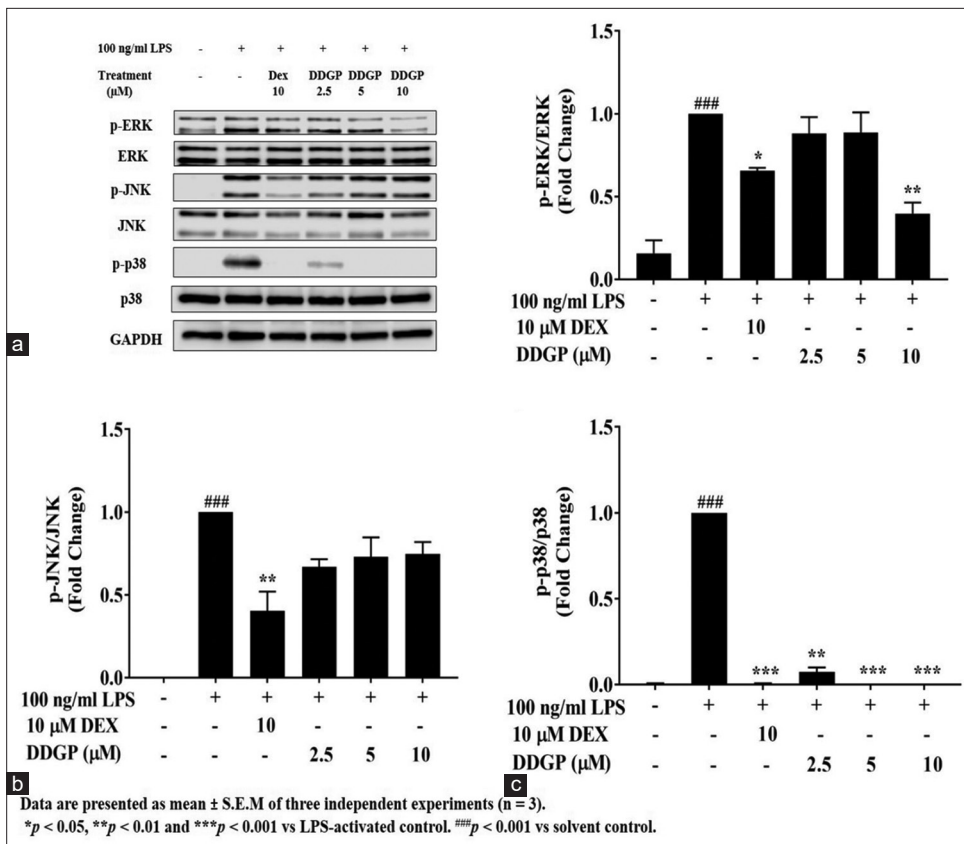
Figure 7: Effects of *S*-deoxydihydroglyparvin on lipopolysaccharide-induced phosphorylation of ERK (a), JNK (b), and p38 MAPK in RAW264.7 cells. GAPDH was used as a loading control
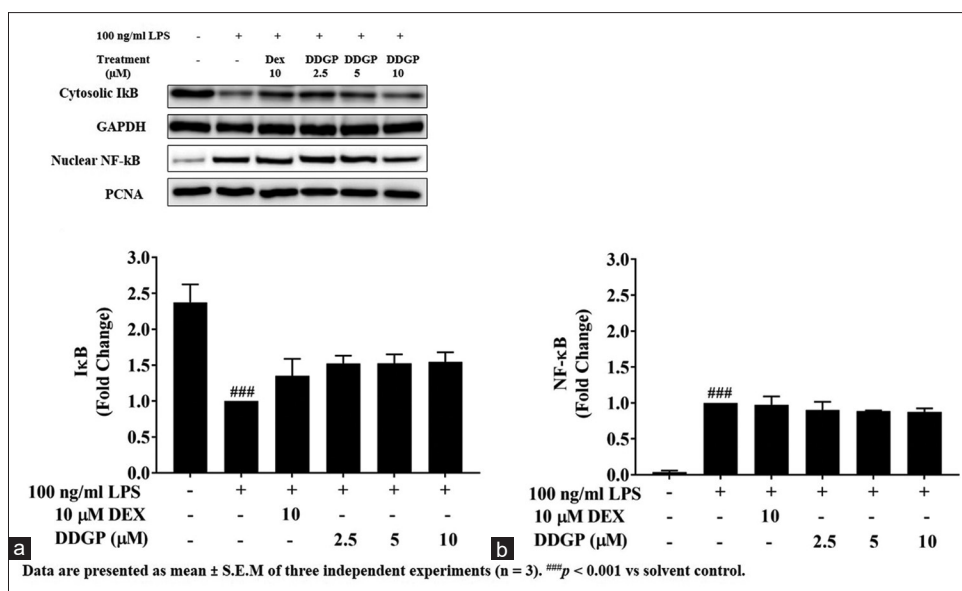
Figure 8: Effects of *S*-deoxydihydroglyparvin on lipopolysaccharide-induced the degradation of cytosolic iκB (a) and the activation of nuclear factor kappa B p65 in the nucleus (b). GAPDH and PCNA were used as loading controls of cytosolic and nuclear proteins, respectively

Figure 9: Proposed mechanisms of anti-inflammatory action of *S*-deoxydihydroglyparvin

amplify the inflammatory process.[18,19] MAPKs are protein serine/threonine kinases with three subtypes: JNK, ERK, and p38 MAPK.[16] Many evidences revealed that activation of MAPKs and NF-κB upregulates pro-inflammatory cytokines and inflammatory mediators.[16,17] Several compounds demonstrated their anti-inflammatory effects through inactivating these pathways.[15,20] DDGP profoundly suppressed phosphorylated p38. It had little or no effect on ERK and JNK phosphorylation and on NF-κB activation.

Several reports suggested that p38 activation involved in inflammation by activating pro-inflammatory cytokines and COX-2 expression.[16,21] A strong link between the p38 activation and many inflammatory diseases was also reported.[22,23] Several natural compounds exhibited anti-inflammatory activities in part by inactivating p38.[15,20] Our results suggest that DDGP may inhibit the early step of LPS-induced macrophage activation through inactivating p38 MAPK, leading to the suppression of the production of inflammation-related cytokines and inflammatory mediators. [Figure 9].

CONCLUSION

We revealed for the first time that DDGP from *G. parva* inhibited LPS-induced macrophage activation by suppressing p38 MAPK activation. DDGP could be considered a potential anti-inflammatory agent.

Conflicts of interest
There are no conflicts of interest.

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