Weigela florida inhibits the expression of inflammatory mediators induced by *Pseudomonas aeruginosa* and *Staphylococcus aureus* infection

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Inflammatory responses involve the action of inflammatory mediators that are necessary for the clearance of invading bacterial pathogens. However, excessive production of inflammatory mediators can damage tissues, thereby impairing bacterial clearance. Here, we examined the effects of *Weigela florida* on the expression of inflammatory cytokines induced by *Pseudomonas aeruginosa* or *Staphylococcus aureus* infection in macrophages. The results showed that pre-treatment with *W. florida* markedly downregulated the bacterial infection-mediated expression of cytokines. Additionally, post-treatment also triggered anti-inflammatory effects in cells infected with *S. aureus* to a greater extent than in those infected with *P. aeruginosa*. Bacterial infection activated inflammation-associated AKT (Thr308 and Ser473)/NF-κB and MAPK (p38, JNK, and ERK) signaling pathways, whereas *W. florida* treatment typically inhibited the phosphorylation of AKT/NF-κB and p38/JNK, supporting the anti-inflammatory effects of *W. florida*. The present results suggest that *W. florida* decreases the infection-mediated expression of inflammatory mediators by inhibiting the AKT/NF-κB and MAPK signaling pathways, implying that it may have potential use as an inhibitory agent of excessive inflammatory responses.

Keywords: anti-inflammation, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Weigela florida*

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Introduction

The complex molecular events underlying innate defense responses are mediated by the action of several inflammatory mediators that are released from recruited immune cells (Medzhitov, 2008). Macrophages are innate immune cells that provide the first-line host defense by sensing invading bacterial pathogens through pattern recognition receptors (PRRs) including toll-like receptors (TLRs) (Akira *et al.*, 2006; Medzhitov, 2010). TLRs, which are expressed on various cell types including immune cells and epithelial cells, recognize conserved microbial structures called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria and peptidoglycans in the cell wall of Gram-positive bacteria (Kumar *et al.*, 2011). This sensing triggers signaling cascades that lead to the production of inflammatory mediators, such as interleukin (IL)-1β, IL-8, and tumor necrosis factor (TNF) α, which regulate host defense responses to infectious agents, thereby promoting the elimination of invading pathogens (Kawai and Akira, 2007). This process is essential for promoting defense mechanisms that mediate the resolution of the acute inflammation, thereby contributing to the recovery of tissue homeostasis (Nathan and Ding, 2010).

However, under certain pathophysiological conditions, excessive cytokine production results in massive immune cell stimulation. This can cause tissue damage to various degrees, of which septic shock is a severe manifestation and one of the most serious health problems worldwide (Liu *et al.*, 2016; Moore and June, 2020). Excessive cytokine production involves the activation of central signaling modulators, such as nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) (Takeuchi and Akira, 2010). Therefore, the signaling pathways activated by these molecules are considered crucial targets for the control of excessive inflammatory responses, which have a dramatic impact on morbidity and mortality, and inhibitors of these molecules are promising drugs to regulate acute inflammatory disorders. The most common therapeutic agents currently in use are steroids, which are effective in suppressing increased inflammatory responses. However, steroids are not considered first-line agents because they are associated with side effects, such as inhibiting the efficacy of anti-tumor therapeutics (Davila *et al.*, 2014). Therefore, control of excessive inflammatory responses remains challenging.

Natural products are a potentially effective therapeutic option for the control of inflammation, and herbal medicines that reduce excessive cytokine production may act by inhibit-

ing the NF-κB pathway. *Weigela* is native to North China, Korea, and Japan, and possesses anticancer properties. *Weigela subsessilis* (*W. subsessilis*) inhibits cancer cell growth and induces apoptosis (Lee *et al.*, 2010). *Weigela florida* upregulates anti-apoptotic genes and tumor suppressor genes to protect human umbilical vein endothelial cells from oxidative stress (Whang *et al.*, 2005). However, the potential effect of *W. florida* on blocking inflammatory cytokine production has not been investigated to date. Here, we examined the effect of *W. florida* and showed that it downregulated the expression of the inflammatory mediators IL-1β, IL-8, and TNFα induced by infection with bacterial pathogens in macrophages, and this effect was mediated by blocking the activation of the AKT/NF-κB and MAPK (p38 and JNK) pathways. These results suggest an anti-inflammatory mechanism underlying the effect of *W. florida* on decreasing inflammatory cytokine production, and support its therapeutic potential for preventing excessive inflammatory responses.

Materials and Methods

Reagents

Escherichia coli (*E. coli*)-derived LPS, the plant extract of *W. florida* (KPM041-046), and pomolic acid were purchased from Sigma-Aldrich, the Korea Plant Extract Bank (KPEB) at the Korea Research Institute of Bioscience and Biotechnology, and Selleckchem, respectively.

Bacterial strains and culture conditions

This study used the *Pseudomonas aeruginosa* (*P. aeruginosa*; Pa) PAO1 wild-type (wt) strain (Holloway, 1955) and the *Staphylococcus aureus* (*S. aureus*; Sa) wt strain (ATCC 25923). Bacteria were grown in Luria (L) broth (0.5% [w/v] yeast extract, 1% [w/v] tryptone, and 1% [w/v] NaCl) or on L agar plates at 37°C. The bacterial cells were harvested at 10,000 × *g* for 1 min at room temperature after overnight broth culture. The bacterial pellet was suspended in phosphate-buffered saline (PBS) for the preparation of live bacteria.

Cell culture

All media described below were supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). RAW 264.7 (mouse macrophage) and RAW-blue (mouse macrophage reporter cells; InvivoGen) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM with high glucose, L-glutamine, and sodium pyruvate; HyClone). THP-1 (human monocyte) cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640; HyClone). Differentiation of THP-1 cells was induced using 50 ng/ml phorbol-12-myristate-13-acetate (PMA) for 48 h, followed by resting for 24 h in the presence of FBS. The resultant cells were designated as dTHP-1 cells in this study. Cells were seeded in 12-well plates (5.0×10^5 cells/well) and incubated at 37°C in a humidified 5% CO_2 air-jacketed incubator for 16 h. After washing to remove non-adherent cells, adherent cells were incubated in FBS-free DMEM for 1 h at 37°C and then infected with either *P. aeruginosa* or *S. aureus* at a multiplicity of infection (MOI) of 1 or 5 at 37°C for 4 h unless specified otherwise.

Alkaline phosphatase assay

RAW-blue cells, a secreted embryonic alkaline phosphatase (SEAP) reporter system, were incubated with various concentrations of *W. florida* extract for 1 h in the presence or absence of LPS and the cell supernatant obtained at 10,000 × *g* for 15 min. SEAP levels were measured by incubating the supernatant with the Quanti-Blue (InvivoGen) substrate for 3 h. Absorption was measured in a SUNRISE microtiter plate reader (Tecan) at 595 nm.

Determination of cell viability

The cell culture media from component-treated cells was analyzed for lactate dehydrogenase (LDH) release using a commercially available kit (CytoTox 96 Non-Radioactive Cytotoxicity assay; Promega). For the MTT assay, cells were incubated with various concentrations of *W. florida* extract (100, 200, and 400 μg/ml) for the indicated times, and cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the TRIzol reagent. The SYBR Green PCR Master Mix (KAPA Biosystems) was used for qRT-PCR. Synthesis of cDNA from total RNA was performed using ReverTra Ace qPCR RT kit (Toyobo). The primer sequences were as follows: mIL-1β primers, 5′-GACCTTCCA GGATGAGGACA-3′ and 5′-AGGCCACAGGTATTTTGT CG-3′; MIP2 primers, 5′-ATCCAGAGCTTGAGTGTGAC GC-3′ and 5′-AAGGCAAACTTTTTGACCGCC-3′; mTNFα primers, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′ and 5′-TGGGAGTAGACAAGGTACAACCC-3′; IL-1β primers, 5′-AAACAGATGAAGTGCTCCTTCCAGG-3′ and 5′-TG GAGAACACCACTTGTTGCTCCA-3′; IL-8 primers, 5′-A ACATGACTTCCAAGCTGGCC-3′ and 5′-TTATGAATT CTCAGCCCTCTTC-3′; TNFα primers, 5′-CAGAGGGAA GAGTTCCCCAG-3′ and 5′-CCTTGGTCTGGTAGGAGA CG-3′. Reactions were performed using a CFX96 Real-Time PCR System (Bio-Rad) using the following thermal conditions: stage 1, 50°C for 2 min and 95°C for 10 min; and stage 2, 95°C for 15 sec and 60°C for 1 min. Stage 2 was repeated for 40 cycles. Relative quantities of mRNA were calculated using the comparative CT method and were normalized against human GAPDH (5′-CCCTCCAAAATCAAGTGG-3′ and 5′-CCATCCACAGTCTTCTGG-3′) and mouse GAPDH (5′-CT CATGACCACAGTCCATGC-3′ and 5′-CACATTGGGGG TAGGAACAC-3′) to determine the amount of RNA used in each reaction.

ELISA

The level of cytokines in culture supernatants was determined by microplate sandwich ELISA using IL-1β ELISA Kits (Thermo), IL-8 ELISA Kits (R&D Systems), and TNFα ELISA Kits (R&D Systems).

Immunoblot analysis

Antibodies were used to analyze total cell lysates according to the manufacturers' instructions. Antibodies against p-AKT (Thr308), p-AKT (Ser473), p-IKKα/β, p-p38, p38, p-ERK, ERK, p-JNK, JNK, and β-actin (D6A8) were purchased from Cell Signaling. Cells were lysed on ice for 10 min in lysis buffer (10 mM Tris-HCl; pH 7.4, 150 mM NaCl, 2.5 mM EDTA, and 0.1% [v/v] Triton-X100) in distilled water (DW) supplemented with 1 mM PMSF (phenylmethylsulfonyl fluoride; Thermo Scientific) and 0.1 mM sodium orthovanadate (Sigma-Aldrich). Samples containing 30 μg of total protein were separated on 8% SDS-PAGE gels and transferred to 0.45 μm polyvinylidene difluoride (PVDF; Millipore Corp.) membranes. Membranes were blocked with 5% (w/v) non-fat dry milk in TBS/0.1% (v/v) Tween 20 (Sigma-Aldrich) at room temperature for 2 h and then probed with primary antibody. Immunoreactive bands were visualized using horseradish peroxidase-linked secondary antibody (Cell Signaling Technology) and enhanced chemiluminescence reagents (Intron Biotechnology).

Statistics

Statistical analyses were performed using the Student's *t*-test or one-way ANOVA followed by Tukey's post-hoc multiple range tests using the Instat package from GraphPad (GraphPad Software). $p < 0.05$ was considered statistically significant.

Results

Weigela florida suppresses LPS-induced NF-κB activation

LPS is a potent macrophage activator that induces the expression of inflammatory mediators by activating NF-κB

(Andreakos *et al.*, 2004). The effect of *W. florida* on LPS-mediated NF-κB activation was examined in RAW-blue cells, which are SEAP reporter cells used for the detection of NF-κB activation. Cells were pre-treated with or without *W. florida* for the indicated times. As shown in Fig. 1A, *W. florida* pre-treatment markedly inhibited LPS-induced NF-κB activity in a dose-dependent manner. To rule out the potential cytotoxicity of the extract, RAW 264.7 macrophages were subjected to an LDH release assay and MTT assay to examine cytotoxicity and cell viability. Exposure of cells to 50, 100, or 200 μg/ml of extract did not increase the release of LDH (Fig. 1B). Treatment with 100, 200, or 400 μg/ml of extract for the indicated times did not affect cell viability (Fig. 1C), indicating no damage to cells. Based on these results, 200 μg/ml was selected as the optimal concentration of *W. florida* extract and used in subsequent experiments. Taken together, these results suggest that *W. florida* suppresses NF-κB activation.

Weigela florida decreases the production of pro-inflammatory cytokines stimulated by bacterial infection

LPS is a representative molecular pattern present in Gram-negative bacteria. To examine the effect of *W. florida* on infection-induced expression of inflammatory cytokines, we measured the levels of *mIL1β*, *MIP2*, and *mTNFα* mRNA in RAW 264.7 cells. Cells were pre-treated with *W. florida* for 1 h, followed by infection with Gram-negative *P. aeruginosa* (MOI 0.5 or 1) for 4 h. As shown in Fig. 2A, the pre-treatment significantly suppressed the infection-induced expression of *mIL1β*, *MIP2*, and *mTNFα*. These results indicate that *W. florida* suppressed the *P. aeruginosa*-induced expression of inflammatory cytokines. Similar results were obtained in cells infected with *S. aureus* (MOI 1 or 5), a representative Gram-positive bacterium, for 4 h (Fig. 2B). To confirm the results, the levels of mRNA and released proteins
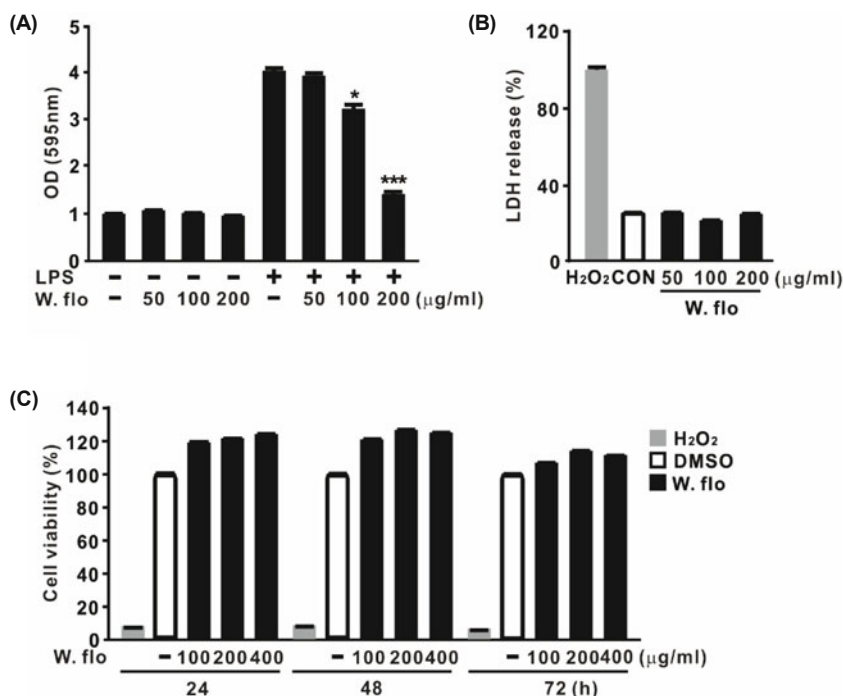

Fig. 1. *Weigela florida* **suppresses LPS-induced NF-κB activation**. (A) RAW-blue cells were seeded in 12-well plates at a concentration of 5×10^5 cells/ml and pre-treated with different concentrations (50, 100, and 200 μg/ml) of *W. florida* extract (W. flo) for 1 h. Cells were then treated with 1,000 ng/ml LPS. LPS-induced NF-κB activation was measured with the alkaline phosphatase assay. (B) RAW 264.7 cells were seeded in 12-well plates at a concentration of 5×10^5 cells/ml and treated with different concentrations (50, 100, and 200 μg/ml) of *W. florida* extract for 24 h. Cytotoxicity was assessed with the LDH release assay. (C) RAW 264.7 cells were treated with different concentrations (100, 200, and 400 μg/ml) of *W. florida* extract for 24, 48, and 72 h, and cell viability was measured with the MTT assay. Data are expressed as the mean ± SD (n = 3). *$p < 0.05$; ***$p < 0.001$ *vs.* treatment with LPS alone (A). DMSO was used as a control (CON).
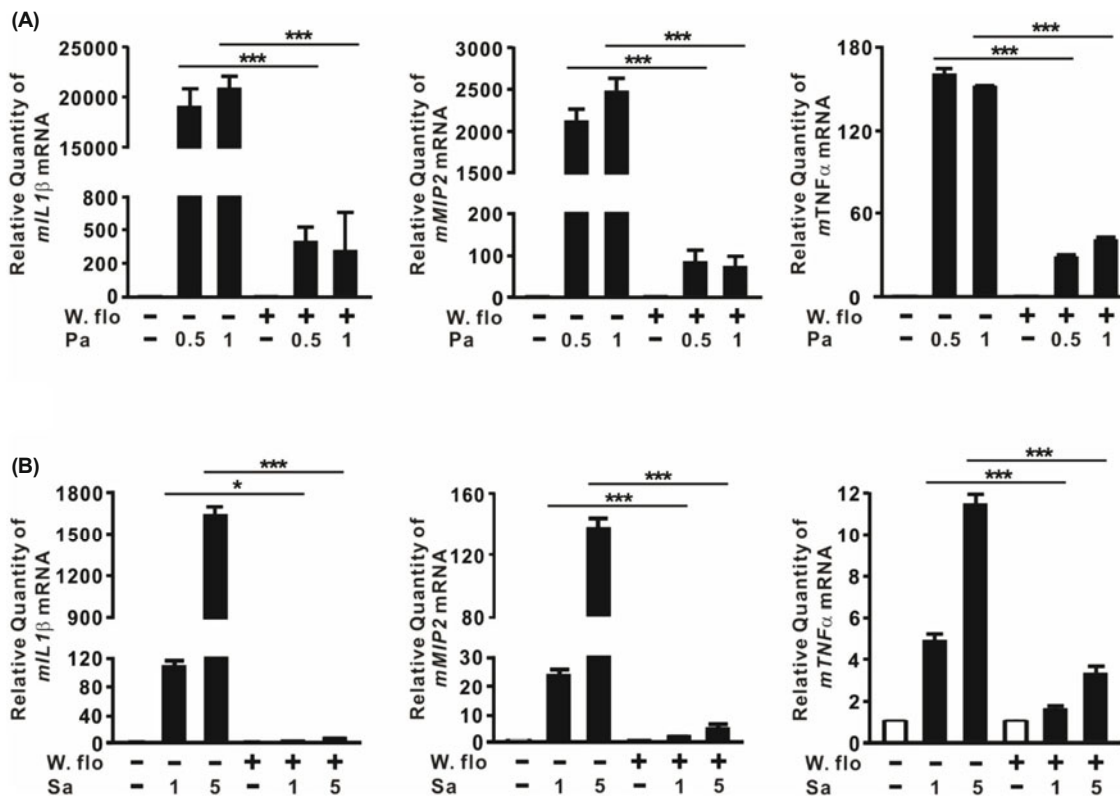
Fig. 2. *Weigela florida* decreases the expression of pro-inflammatory cytokines stimulated by bacterial infection in RAW 264.7 cells. RAW 264.7 cells were pretreated with 200 µg/ml of *W. florida* extract for 1 h followed by treatment with either *P. aeruginosa* (Pa) or *S. aureus* (Sa) at a multiplicity of infection (MOI) of 0.5 and 1 for Pa (A) or 1 and 5 for Sa (B). The mRNA levels of *mIL1β*, *MIP2*, and *mTNFα* were measured by qRT-PCR. Data are expressed as the mean ± SD (n = 3). *$p < 0.05$; ***$p < 0.001$ *vs.* no pre-treatment with *W. florida* extract.

were examined in dTHP-1 human macrophages, which are used as a common model to examine the modulation of macrophage-related activities, such as signaling pathways and defense mechanisms to invading microbes. The cells were pretreated with *W. florida* extract for 1 h, followed by infection with *P. aeruginosa* (MOI 1) or *S. aureus* (MOI 5). In addition, the cells were post-treated with *W. florida* extract after infection with bacterial strains for 1 or 2 h. Pretreatment with *W. florida* decreased cytokine mRNA (Fig. 3A and B) and protein levels in macrophages (Fig. 3C and D). Similar to the effects of pre-treatment, cytokine mRNA expression remained low after post-treatment, although the post-treatment effect on protein expression was lower than that of pre-treatment. These data demonstrate that not only pre-treatment, but also post-treatment with *W. florida* decreases the expression of pro-inflammatory cytokines induced by bacterial infection.

Weigela florida inhibits the activation of the AKT/NF-κB and MAPK pathways in response to bacterial infection

Infection-induced expression of inflammatory mediators is associated with the activation of NF-κB, which is linked to the activity of AKT and MAPKs (including extracellular signal-regulated kinase [ERK], c-Jun N-terminal kinase [JNK], and p38) (Bava *et al.*, 2011). Because these signaling pathways are targets of anti-inflammatory molecules, we examined the effects of pre-treatment with *W. florida* on the activity of these

regulators in response to bacterial infection. As shown in Fig. 4A, AKT phosphorylation at both Thr 308 and Ser 473 increased gradually starting at 15 min after infection with *P. aeruginosa* and began to decrease after 60 min. In response to infection of *S. aureus*, AKT phosphorylation increased after 30 min and continued to increase until 120 min (Fig. 4B). Unlike the activation of AKT, phosphorylation of IKKα/β was observed after 60 min of exposure to each pathogen and decreased thereafter, suggesting that NF-κB activation was under the control of AKT. *Weigela florida* pre-treatment significantly suppressed infection-induced AKT and NF-κB activation. The decrease in AKT phosphorylation was greater at Thr 308 than at Ser 473. Phosphorylation of MAPKs increased after 30 min of exposure to each pathogen (Fig. 4C and D). *Weigela florida* pre-treatment suppressed the infection-induced increase in the phosphorylation of p38 and JNK, whereas it had no significant effect on ERK phosphorylation, suggesting that the suppressive effects of the extract were limited to the activation of p38 and JNK. Taken together, these results suggest that *W. florida* suppresses the *P. aeruginosa* or *S. aureus*-mediated activation of AKT/NF-κB and p38/JNK MAPKs.

Effect of pomolic acid on the regulation of pro-inflammatory cytokines in response to bacterial infection.

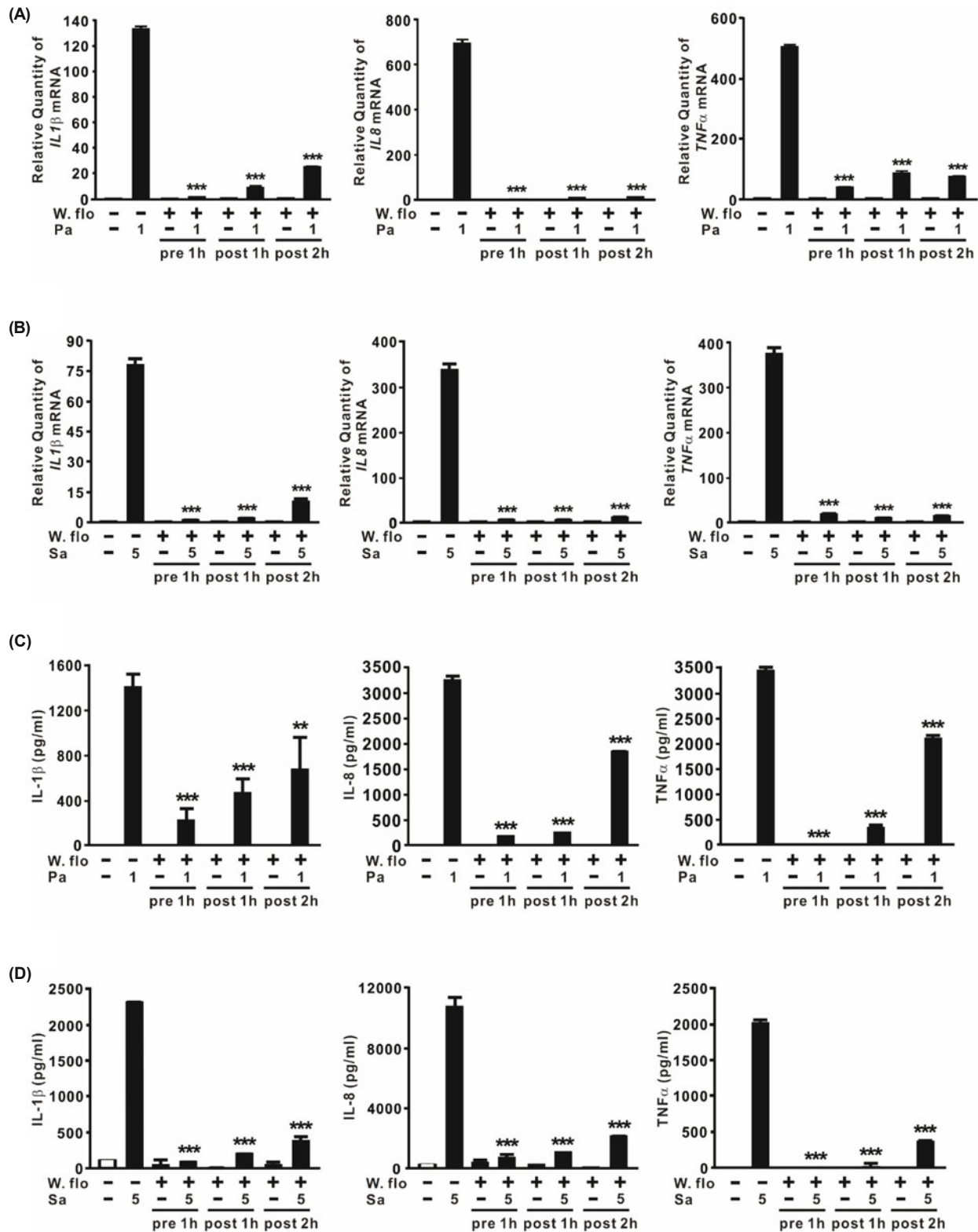The results showing that *W. florida* treatment decreases cy-

Fig. 3. *Weigela florida* **decreases the production of pro-inflammatory cytokines stimulated by bacterial infection in dTHP-1 cells.** dTHP-1 cells were pretreated with 200 μg/ml of *W. florida* extract for 1 h or post-treated for 1 or 2 h followed by treatment with *P. aeruginosa* (Pa) or *S. aureus* (Sa) at an MOI of 1 for Pa (A and C) or 5 for Sa (B and D). mRNA and protein levels were measured by qRT-PCR and ELISA. Data are expressed as the mean ± SD (n = 3). **$p < 0.01$; ***$p < 0.001$ *vs.* no pre-treatment with *W. florida* extract.

tokine expression induced by bacterial infection prompted us to investigate the effect of pomolic acid, an anti-inflammatory component of the extract of *W. subsessilis* (Thuong *et al.*, 2005). dTHP-1 cells were pre-treated with 50 mM of pomolic acid for 1 h before bacterial infection or post-treated with the same concentration of pomolic acid for 1 or 2 h after bacterial infection. Pre-treatment with pomolic acid did not decrease bacterial-induced cytokine mRNA levels (Fig. 5A and B). By contrast, post-treatment with pomolic acid decreased bacterial-induced cytokine mRNA levels. However, the effect was not as strong as that observed with the *W. florida* extracts (Fig. 3A and B), suggesting that pomolic acid is not the component responsible for the inhibitory effects of *W. florida* extracts. However, because cytokine expression decreased in response to post-treatment, we examined the phosphorylation of signaling mediators to compare the inhibitory effect of pomolic acid with that of the extract. As shown in Fig. 5C and D, the degree of phosphorylation did not change significantly in response to pre-treatment with pomolic acid, indicating that pomolic acid might not mediate the inhibitory effect of *W. florida* extract.

Discussion

In this study, we demonstrated that the *W. florida* extract decreases the expression of the inflammatory mediators IL-1β, IL-8, and TNFα induced by infection with *P. aeruginosa* and *S. aureus*, two multi-drug resistant Gram-negative and -positive bacteria, respectively (Fig. 3), by suppressing the activation of AKT/NF-κB and MAPK (p38 and JNK) signaling in macrophages (Fig. 4). This effect was not due to an inhibitory effect of the *W. florida* extract on cell viability because the LDH release and MTT cell viability assays showed that the amounts of *W. florida* extract used in this study were not cytotoxic (Fig. 1B and C).

Pre-treatment with *W. florida* significantly inhibited LPS-induced NF-κB activation (Fig. 1A) and markedly decreased the infection-induced mRNA and protein expression of inflammatory mediators as shown in Fig. 3. The anti-inflammatory effect of *W. florida* remained active post-treatment, as indicated by the reduced expression levels of inflammatory mediators in infection-stimulated macrophages, although the inhibitory effect decreased in a time-dependent manner. The negative effect of post-treatment (especially 2 h) with the *W. florida* extract on the expression of the inflammatory mediators at the protein level was greater in cells exposed to *S. aureus* than in those exposed to *P. aeruginosa* (Fig. 3C and D), suggesting that the extract is more effective in interfering with inflammatory signaling triggered by *S. aureus* infection than in interfering with those triggered by *P. aeruginosa*. Another possible explanation is that *P. aeruginosa* has a stronger effect on stimulating inflammatory signaling than *S. aureus*. To further investigate the effect of *W. florida* on the activation of signaling pathways, we examined the activation of inflammatory signaling mediators. As shown in Fig. 4, bacterial infection activated the AKT/NF-κB and MAPK signaling pathways, and pre-treatment with *W. florida* markedly suppressed the phosphorylation of signaling molecules except ERK in dTHP-1 cells, indicating that *W. florida* was almost equally effective in inhibiting signaling initiated by both bacterial pathogens. However, p38 and JNK phosphorylation in response to *S. aureus* was weaker than that caused by *P. aeruginosa*, which could explain the stronger effects of *W. florida* on the activation of signaling in response to *S. aureus* shown in Fig. 3D. Currently, the mechanism via which *W. florida* inhibits kinase activity is unclear. It is possible that the *W. florida* extract may act on several kinases because the extract reduced the activation of not only Akt/NF-κB but also MAPKs. Therefore, the extract may modulate the activity of upstream signaling mediators governing the expression of inflammatory signaling mediators. Interestingly,
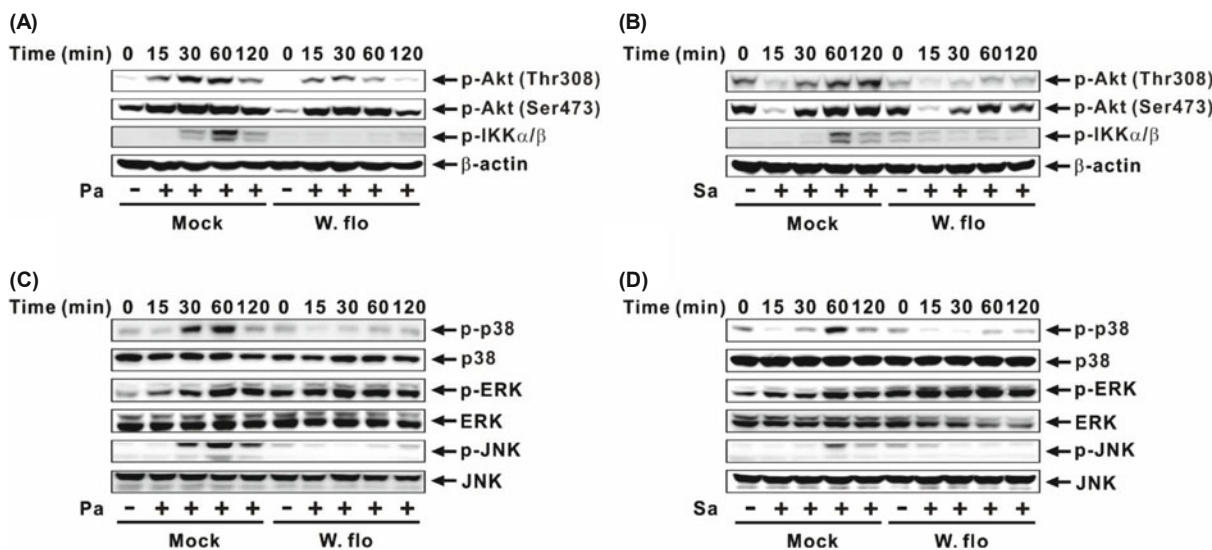

Fig. 4. *Weigela florida* **inhibits the activation of the AKT/NF-κB and MAPK pathways in response to bacterial infection.** dTHP-1 cells were pre-treated with 200 µg/ml of *W. florida* extract for 1 h followed by treatment with *P. aeruginosa* (Pa) or *S. aureus* (Sa) at an MOI of 1 for Pa (A and C) or 5 for Sa (B and D). The activation levels were examined by immunoblot analysis. Immunoblot data are representative of three separate experiments.
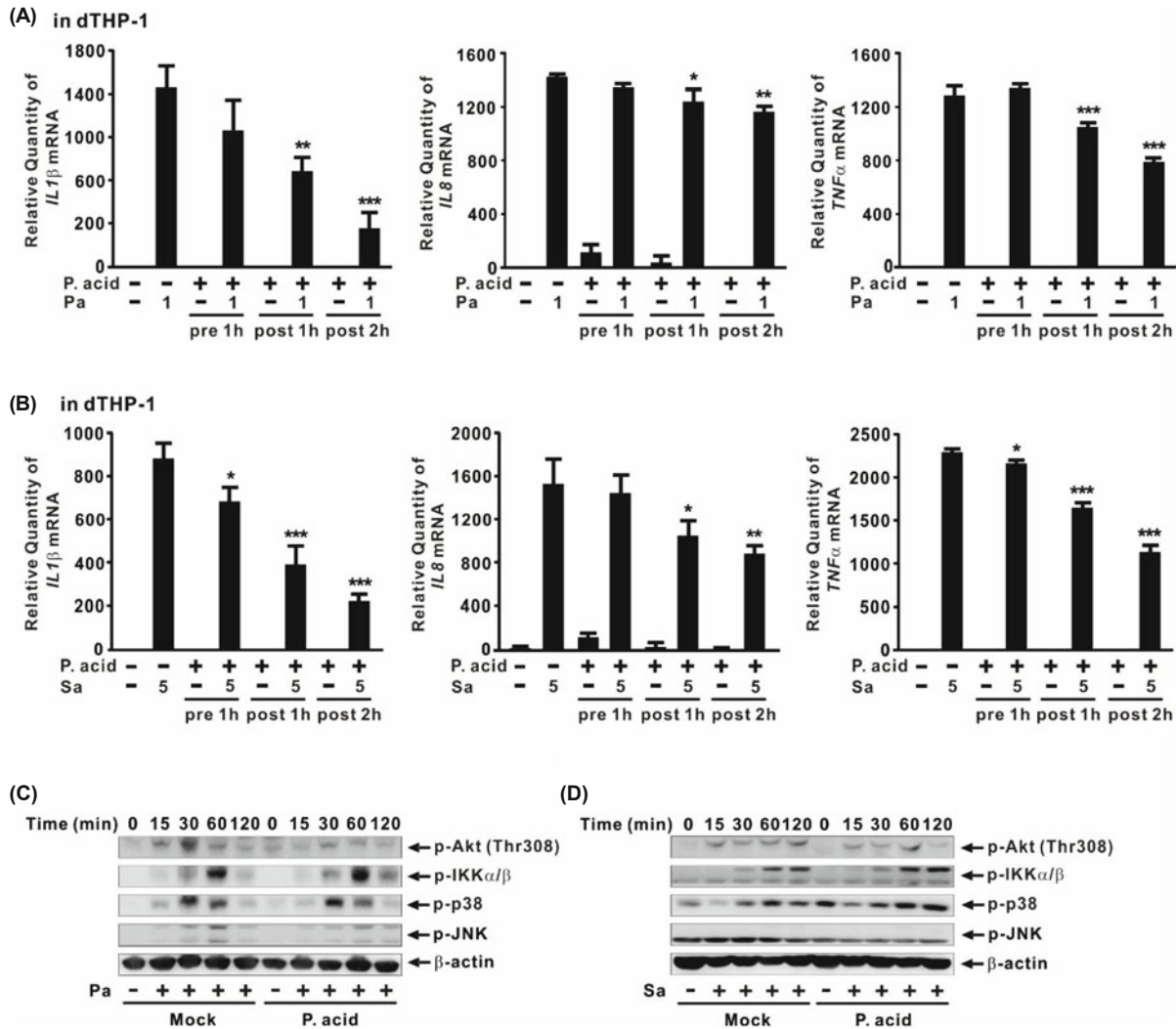
(A) in dTHP-1

(B) in dTHP-1

(C)

(D)

Fig. 5. **Effect of pomolic acid on the regulation of pro-inflammatory cytokines in response to bacterial infection**. (A and B) dTHP-1 cells were pre-treated with 50 mM of pomolic acid for 1 h or post-treated for 1 or 2 h followed by treatment with *P. aeruginosa* (Pa) or *S. aureus* (Sa) at an MOI of 1 for Pa (A) or 5 for Sa (B). mRNA levels were measured by qRT-PCR. (C and D) dTHP-1 cells were pre-treated with 50 mM of pomolic acid for 1 h followed by treatment with Pa or Sa at an MOI of 1 for Pa (C) or 5 for Sa (D). The activation levels were examined by immunoblot analysis. Immunoblot data are representative of three separate experiments. Data are expressed as the mean ± SD (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs.* no pretreatment with pomolic acid. DMSO was used as a control (CON).

we observed a decrease in the level of phosphorylated Akt at 15 min after infection with *S. aureus* (Fig. 4B) but not after infection with *P. aeruginosa*, indicating that the inhibitory effect may be bacterial-specific. According to a previous report, α-toxin from *S. aureus* can markedly attenuate Akt activation in a dose-dependent manner (Wiles *et al.*, 2008), suggesting that this could be one mechanism via which sublytic concentrations of bacterial toxins can modulate inflammatory responses during infection.

The present results suggest that *W. florida* markedly inhibits bacterial infection-induced inflammation. Because the active components of *W. florida* extracts have not yet been identified or isolated, it is unclear which components are responsible for its ability to attenuate the effects of bacterial infection. Consistent with the inhibitory effect of *W. florida*, *W. subsessilis*, a close relative of *W. florida*, has also been

reported to strongly inhibit IL-8 expression in TNFα-stimulated HT29 cells (Thuong *et al.*, 2005). *Weigela subsessilis* extracts have been shown to contain twelve components: three sterols (J3-sitosterol acetate, 13-sitosterol, daucosterol), eight triterpenoids (squalene, ursolic acid, ilekudinol A, corosolic acid, ilekudinol B, esculentic acid, pomolic acid, and asiatic acid), and one iridoid glycoside (alboside I) (Thuong *et al.*, 2005). Among these, three compounds, namely ilekudinol B, pomolic acid, and alboside I, strongly inhibit the expression of IL-8 in HT29 cells. Thus, we compared the effect of pomolic acid with that of *W. florida* extracts, especially as it can be isolated from a broad spectrum of plants and is commercially available. However, the effect of pomolic acid was not comparable with that of the extract, although post-treatment with pomolic acid decreased the expressions of cytokines to some extent (Fig. 5). In Fig. 5C and D, the increase in the levels

of inflammatory mediators upon treatment with bacteria was weak. This could be due to the status of the cells, which had been sub-cultured to a relatively high passage number. Although the levels were relatively weak, they were sufficient to conclude that treatment with pomolic acid does not results in the same level of inhibition of inflammatory mediators as that observed with the *W. florida* extract. This implies that the underlying inhibitory mechanism of pomolic acid is different from that of *W. florida*. However, the components of the *W. florida* extract may play diverse roles in this inhibitory process by acting on different downstream molecules of the inflammatory signaling pathway stimulated by infection. Therefore, we can-not rule out the possibility that pomolic acid, as one of the components of *W. florida*, may modulate other signaling mediators in different ways.

In summary, this study demonstrated that *W. florida* suppresses bacterial infection-induced production of inflammatory cytokines in dTHP-1 cells by inhibiting the activation of the AKT/NF-κB and MAPKs (p38 and JNK) signaling pathways, although the possibility that other pathways are involved could not be excluded. Excessive inflammatory responses in the human body, such as the cytokine storm, cause clinically life-threatening conditions manifested by extremely elevated serum cytokine levels, vascular leakage, and thrombosis, leading to multisystem organ dysfunction. The present results thus suggest the therapeutic potential of *W. florida* for the treatment of tissue damage caused by an excessive inflammatory response, as well as its potential use as an anti-inflammatory agent to prevent inflammatory diseases from adversely affecting macrophages. Further study is necessary to identify the components of *W. florida* extract responsible for its effects, which may help modulate excessive inflammatory responses by controlling the level of cytokines. These components could be useful for the treatment of inflammatory disorders in combination with other anti-inflammatory drugs.

Acknowledgements

Conflict of Interest

The authors have no conflict of interest to report.

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