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# HangAmDan-B, an Ethnomedicinal Herbal Mixture, Suppresses Inflammatory Responses by Inhibiting Syk/NF-κB and JNK/ATF-2 Pathways

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ABSTRACT HangAmDan-B (HAD-B) is a powdered mixture of eight ethnopharmacologically characterized folk medicines that is prescribed for solid masses and cancers in Korea. In view of the finding that macrophage-mediated inflammation is a pathophysiologically important phenomenon, we investigated whether HAD-B modulates inflammatory responses and explored the associated molecular mechanisms. The immunomodulatory activity of HAD-B in toll-like receptor-activated macrophages induced by lipopolysaccharide (LPS) was assessed by measuring nitric oxide (NO) and prostaglandin $E_2$ ($PGE_2$) levels. To identify the specific transcription factors (such as nuclear factor [NF]–κB and signaling enzymes) targeted by HAD-B, biochemical approaches, including kinase assays and immunoblot analysis, were additionally employed. HAD-B suppressed the production of $PGE_2$ and NO in LPS-activated macrophages in a dose-dependent manner. Furthermore, the extract ameliorated HCl/EtOH-induced gastritis symptoms. Moreover, HAD-B significantly inhibited LPS-induced mRNA expression of inducible NO synthase and cyclooxygenase (COX)-2. Interestingly, marked inhibition of NF-κB and activating transcription factor was observed in the presence of HAD-B. Data from direct kinase assays and immunoblot analysis showed that HAD-B suppresses activation of the upstream signaling cascade involving spleen tyrosine kinase, Src, p38, c-Jun N-terminal kinase, and transforming growth factor β–activated kinase 1. Finally, kaempferol, but not quercetin or resveratrol was identified as a bioactive compound in HAD-B. Therefore, our results suggest that HAD-B possesses anti-inflammatory activity that contributes to its anticancer property.

**KEY WORDS:** • ***ATF-2*** • ***HangAmDan-B*** • ***inflammation*** • ***macrophages*** • ***NF-κB*** • ***p38*** • ***Syk***

## INTRODUCTION

INFLAMMATION IS A natural mode of defense against foreign materials in the human body. However, inflammatory responses occasionally trigger a serious condition leading to diseases, such as cancer, diabetes, atherosclerosis, and arthritis.[1] Such inflammatory events are mostly managed by macrophages. These cells overproduce numerous soluble factors, including pro-inflammatory cytokines (tumor necrosis factor [TNF]-α and interleukin [IL]-1] and inflammatory molecules (nitric oxide [NO] and prostaglandins [PG]), that contribute to the onset of inflammatory responses and disease.[2] Considerable efforts to date have facilitated our understanding of the molecular inflammatory events by which bacterial or viral products trigger molecular interactions between immunogens, pattern recognition receptors (*e.g.*, toll-like receptor [TLR]) and their adaptor molecules (*e.g.*, TIR-domain–containing adapter-inducing interferon-β [TRIF] and myeloid differentiation primary response gene (88) [MyD88]). For instance, lipopolysaccharide (LPS)/TLR4 interactions activate protein kinases, such as Src, spleen tyrosine kinase (Syk), phosphoinositide 3-kinase (PI3K), and Akt (protein kinase B), as well as mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. The signaling cascades are eventually linked to the activation of inflammation-regulating transcription factors, such as nuclear factor (NF)–κB, activating transcription factor (ATF)–2, and activator protein (AP)–1.[3,4] Targeting of specific signaling events or steps in the inflammatory response pathway is an important strategy for the development of anti-inflammatory drugs and treatment of inflammation-mediated diseases, such as cancer and atherosclerosis.[5]

HangAmDan-B (HAD-B), consisting of Radix *Panax notoginseng*, *Cordyceps militaris*, *Cremastra appendiculata*, Radix *Panax ginseng*, calculus bovis, *Ipomoea batatas*

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'Margarita', *Boswellia carteri*, and *Commiphora myrrha*, is an upgraded version of HAD used traditionally for solid masses, as discussed in the classical texts ''Xi Huang Wan'' and ''Wai Ke Zheng Zhi Quan Sheng Ji.''[6–11] A mixture of these plants has been shown to exert strong anticancer activity against solid tumors, including pancreatic, lung, colorectal, and stomach cancers.[12] Additionally, antiangiogenesis effects and inhibition of cancer cell proliferation and metastasis have been reported.[13] In particular, case reports observed with HAD have been selected as part of the National Cancer Institute's Best Case Series Program.[14] HAD-B has shown efficacy in inhibiting migration and proliferation of human umbilical vein endothelial cells and limiting the formation of capillary tube structures.[15] Furthermore, safety evaluations of HAD-B have revealed no side-effects in either healthy subjects or cancer patients.[16]

In view of the finding that inflammation is a basic pathophysiological phenomenon in tumorigenic responses to specific cancer cells and tissues, an anti-inflammatory approach has been suggested for cancer therapy.[17,18] In particular, pro-inflammatory roles of macrophages are critical in cancer cell survival, proliferation, migration, and metastasis, and thus, regulation of macrophage functions has been considered as a therapeutic strategy.[19] In this respect, it is important to determine whether HAD-B displays modulatory activity against macrophage-mediated inflammatory responses. To address this issue, we investigated the anti-inflammatory effect of HAD-B on activated macrophages induced by the TLR4 ligand, LPS, by measuring the levels of NO and $PGE_2$, and examining the molecular events involved in LPS-mediated inflammatory responses.

## MATERIALS AND METHODS

### Materials

Ranitidine, sodium carboxylmethylcellulose (CMC), and LPS was obtained from Sigma Chemical Co. (St. Louis, MO, USA). SB203580, SP600125, BAY61-3606, and piceatannol were purchased from Calbiochem (La Jolla, CA, USA), and RAW264.7 cells from ATCC (Rockville, MD, USA). All other chemicals were of reagent grade. Anti-phospho or total antibodies against Src, Syk, ERK, p38, JNK, transforming growth factor $\beta$–activated kinase 1 (TAK1), mitogen-activated protein (MAP) kinase kinase (MKK)3/6, MKK4, p65 and p50 (NF-$\kappa$B), ATF-2, p85/PI3K, inhibitors of $\kappa$B$\alpha$ (I$\kappa$B$\alpha$), I$\kappa$B$\alpha$ kinase (IKK), AP-1 (c-Jun, c-Fos, and Fra-1), $\beta$-actin, and $\gamma$-tubulin were obtained from Cell Signaling (Beverly, MA, USA).

### Preparation of HAD-B

HAD-B (Dunsan Oriental Hospital, Daejeon, Korea), an herbal formula consisting of Radix *P. notoginseng*, *C. militaris*, *C. appendiculata*, Radix *P. ginseng*, calculus bovis, *I. batatas* 'Margarita', *B. carteri*, and *C. myrrha* in powder form, was prepared as reported previously[12,13,16] and stored at −20°C until use.

### Mice

Six-week-old male ICR mice were purchased from B&K (Fremont, CA, USA). Mice were given food pellets (Samyang, Daejeon, Korea) and water *ad libitum* under a 12-h light–12-h dark cycle. Studies were performed in accordance with guidelines established by the Sungkyunkwan University Institutional Animal Care and Use Committee.

### Cell culture

RAW264.7 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, USA), glutamine, and antibiotics (penicillin and streptomycin) at 37°C with 5% $CO_2$. For each experiment, cells were detached with a cell scraper. Experiments were performed with a cell density of $2\times10^6$ cells/mL. At this density, more than 99% of cells were viable, as evidenced by Trypan blue staining.[20]

### Treatment with HAD-B

The stock solution (100 mg/mL) of HAD-B was suspended in 1 mL of dimethyl sulfoxide (DMSO) and sonicated for 6 h. After centrifugation, the DMSO soluble layer was prepared. Based on previous studies, noncytotoxic concentrations (0–200 $\mu$g/mL) of HAD-B were prepared by dilution with RPMI 1640 for *in vitro* experiments. For gastritis experiments, 200 mg/kg HAD-B suspended with 1% CMC solution was used for oral administration.

### NO and $PGE_2$ production

After RAW264.7 cells ($1\times10^6$ cells/mL) were incubated for 18 h, cells were pretreated with HAD-B (0–200 $\mu$g/mL) for 30 min. Next, cells were stimulated with LPS and incubated for a further 24 h. The inhibitory effects of HAD-B on NO and $PGE_2$ production were determined by analyzing their levels using Griess reagent, and enzyme immunoassay kits, respectively, as described previously.[21,22]

### MTT assay

Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described in a previous report.[23,24]

### Semiquantitative reverse transcription–polymerase chain reaction for mRNA detection

Total RNA from LPS-treated-RAW264.7 cells ($5\times10^6$ cells/mL) was prepared using TRIzol Reagent (Gibco BRL), according to the manufacturer's protocol.[25,26] Total RNA was stored at −70°C until use. Semiquantitative reverse transcription reactions were conducted using MuLV reverse transcriptase. Specifically, total RNA (1 $\mu$g) was incubated with oligo-dT15 for 5 min at 70°C and mixed with 5× first-strand buffer, 10 mM dNTPs, and 0.1 M DTT. The reaction mixture was further incubated for 5 min at 37°C and 60 min after the addition of MuLV reverse transcriptase (2 U). Reactions were terminated by heating for 10 min at 70°C.

Table 1. Reverse Transcription–Polymerase Chain Reaction Primers Used in This Experiment

| *Name* | *Sequence (5′ to 3′)* |
|---|---|
| iNOS | F: GGA GCC TTT AGA CCT CAA CAG A<br>R: TGA ACG AGG AGG GTG GTG |
| TNF-α | F: TGC CTA TGT CTC AGC CTC TTC<br>R: GAG GCC ATT TGG GAA CTT CT |
| COX-2 | F: CACTACATCCTGACCCACTT<br>R: ATGCTCCTGCTTGAGTATGT |
| GAPDH | F: CAA TGA ATA CGG CTA CAG CAA C<br>R: AGG GAG ATG CTC AGT GTT GG |

iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; COX, cyclooxygenase.

Total RNA was depleted by adding RNase H. Polymerase chain reaction (PCR) were conducted using the following conditions: 2 $\mu$L cDNA, 4 $\mu$M 5′ and 3′ primers, 10× buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 0.1% Triton X-100), 250 $\mu$M dNTPs, 25 mM $MgCl_2$, and 1 unit of *Taq* polymerase (Promega, Madison, WI, USA). The following conditions were used for amplification: 30 sec denaturation at 94°C, 30 sec annealing between 55 and 60°C, 45 sec extension at 72°C, and a final extension step of 5 min at 72°C. The primers (Bioneer, Daejeon, Korea) utilized in this experiment (Table 1) were used as reported previously.[21,23]

### *Total lysate and nuclear extract preparation and immunoblot analysis*

Total lysates and nuclear extracts from LPS-treated RAW264.7 cells pretreated with HAD-B were prepared according to a previously published method.[24,27] Immunoblot analyses of phospho- or total levels of transcription factors (p65, p50, c-Jun, c-Fos, Fra-1, and ATF-2), MAPK (ERK, p38, and JNK), MAP ERK kinase (MEK) 1/2, MKK3/6, MKK4, TAK1, IL-1 receptor-associated kinase (IRAK) 1, I$\kappa$B$\alpha$, IKK$\beta$, p85/PI3K, $\gamma$-tubulin, and non-receptor tyrosine kinases (Src and Syk) were performed according to previously published methods.[25]

### *Syk and Src kinase assays*

To evaluate Syk and Src kinase inhibitory activities with purified enzymes, a kinase profiler service from Millipore was employed. Human Src or Syk (1–5 mU) was incubated with reaction buffer in a final reaction volume of 25 $\mu$L. Reactions were initiated by the addition of MgATP. After 40-min incubation at room temperature, reactions were terminated by adding 5 mL of 3% phosphoric acid solution. Reaction products (10 $\mu$L) were spotted onto a P30 filtermat, which was washed three times in 75 mM phosphoric acid and once in methanol for 5 min each before drying and scintillation counting.

### *EtOH/HCl-induced gastritis*

Stomach inflammation was induced with EtOH/HCl, according to a published method.[28,29] Fasted ICR mice were orally treated with HAD-B (200 mg/kg) or ranitidine (40 mg/kg) for 3 times (twice/day). Thirty minutes later after the last administration, 400 $\mu$L of 60% ethanol in 150 mM HCl was administered orally. Each animal was anesthetized with an overdose of urethane 1 h after the administration of necrotizing agents. The stomach was then excised and gently rinsed under running tap water. After opening the stomach along the greater curvature and spreading it out on a board, the area (in $mm^2$) of mucosal erosive lesions was measured with a ruler and photographed. The gastric lesion after inducer alone was considered 100%.

### *High-performance liquid chromatography analysis*

Phytochemical characteristics of HAD-B and the standard compounds resveratrol, kaempferol, and quercetin were identified by high-performance liquid chromatography (HPLC) analysis.[30] The system was equipped with a model K-1001 HPLC pump, model K-2600 fast scanning spectrophotometer, and a model K-500 4-channel degasser (all from KNAUER Wellchrom, Berlin, Germany). Elution solvents were buffer A (0.1% trifluoroacetic acid [TFA] in $H_2O$) and buffer B (0.08% TFA in 95% acetonitrile (can) + 5% $H_2O$). The gradient step of the solvent was buffer A to buffer B per minute, and a Phenomenex Gemini $C_{18}$ ODS (5 $\mu$m) column was used as reported previously.[31]

### *Statistical analysis*

For statistical comparison, results were assessed using analysis of variance/Scheffe's *post hoc* test and Kruskal–Wallis/Mann–Whitney tests. $P$ values < .05 were considered statistically significant. All statistical tests were carried out using the SPSS program (SPSS Inc., Chicago, IL, USA). Data were obtained from at least three independent experiments performed in triplicate, and presented as means ± standard error of the mean. The other data are representative of three different experiments yielding similar results.

## RESULTS AND DISCUSSION

In view of the pathophysiological role of macrophages in tumorigenic responses and the utility of HAD-B as an ethnopharmacological medicine for cancer,[14,17] we further investigated whether the formulation decreases macrophage-mediated inflammatory responses. Accordingly, the anti-inflammatory activity of HAD-B was examined using TLR-activated macrophages and molecular targets.

HAD-B blocked the production of NO (Fig. 1A) and $PGE_2$ (Fig. 1B) from RAW264.7 cells under LPS stimulation in a dose-dependent manner. This inhibition by HAD-B does not appear to be due to its nonspecific suppressive effect, since no dramatic decrease in cell viability was observed upon treatment with HAD-B up to a concentration of 200 $\mu$g/mL (Fig. 1C). Analysis of mRNA levels (Fig. 1D) of inducible NO synthase (iNOS), a NO-producing enzyme, and cyclooxygenase (COX)-2, a $PGE_2$-producing enzyme, revealed HAD-B-mediated inhibition of NO and $PGE_2$

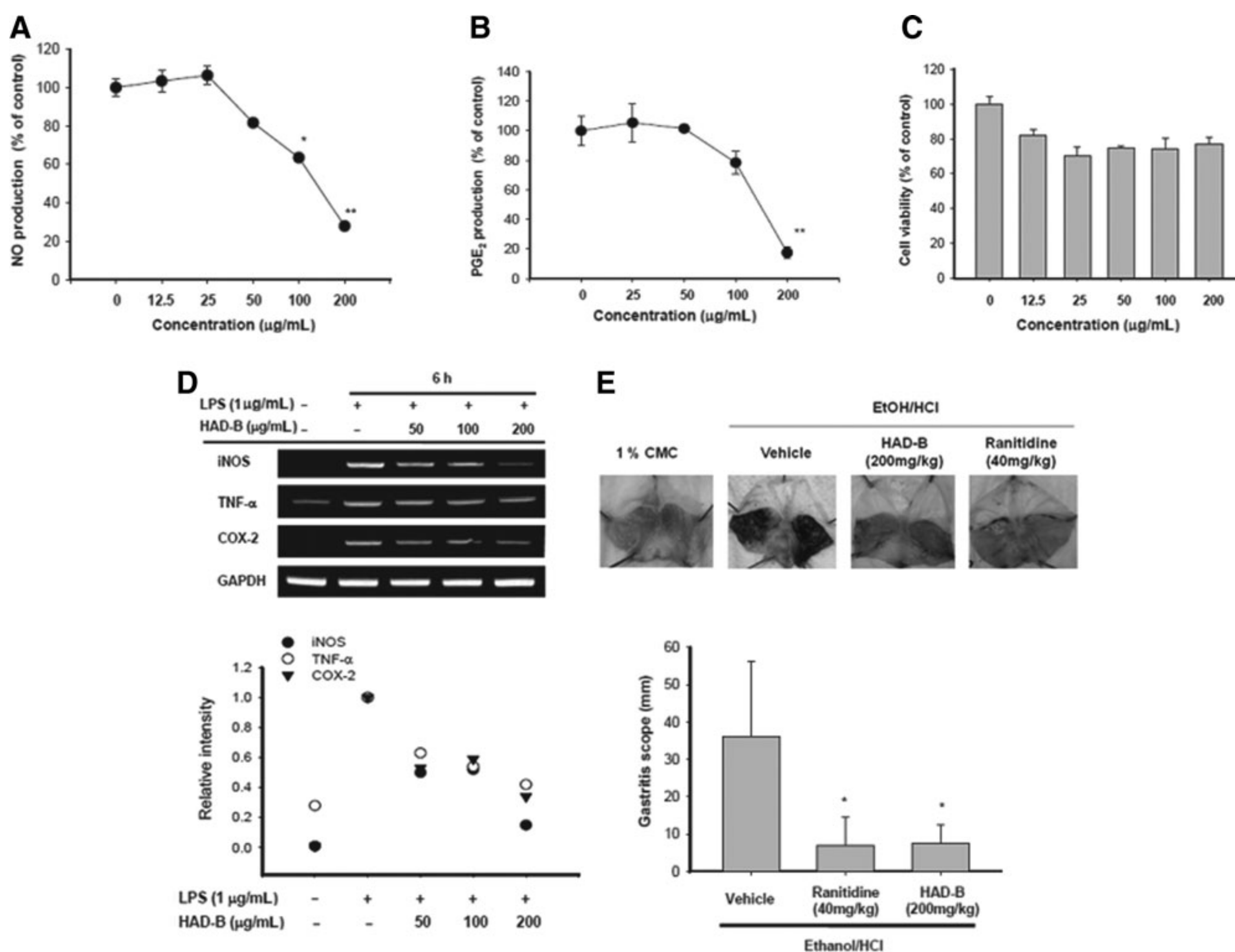


**FIG. 1.** Effect of HAD-B on inflammatory responses *in vitro*. **(A, B)** To examine the anti-inflammatory effects *in vitro*, NO and $PGE_2$ levels in culture supernatants prepared from LPS-activated RAW264.7 cells pretreated with HAD-B were determined with Griess reagent and EIA. **(C)** The viability of RAW264.7 cells pretreated with HAD-B was determined with the MTT assay. **(D)** iNOS, TNF-α, and COX-2 mRNA levels from LPS-activated RAW264.7 cells pretreated with HAD-B were determined using semiquantitative RT–polymerase chain reaction. **(E)** Mice orally administered with HAD-B for 3 times (twice/day) were orally treated with EtOH/HCl. After 1 h of fourth treatment, gastric lesions in the stomach were measured with a ruler (*bottom*) and photos of these were taken by a camera (*top*). The gastric lesion after inducer alone is represented by 100%. Relative intensity of bands was measured using densitometry scanning. $^{*}P<.05$ and $^{**}P<.01$, compared to the control group. HAD-B, HangAmDan-B; NO, nitric oxide; $PGE_2$, prostaglandin $E_2$; LPS, lipopolysaccharide; iNOS, inducible NO synthase; TNF, tumor necrosis factor; COX, cyclooxygenase; EIA, enzyme immunoassay.

production at the transcriptional level. The inhibitory activity of HAD-B was also confirmed *in vivo* using a gastritis model. Thus, this extract (200 mg/kg) strongly ameliorated EtOH/HCl-induced gastric lesions similarly to the standard compound ranitidine (Fig. 1E), confirming HAD-B effectiveness both *in vitro* and *in vivo*.

Since LPS-induced macrophage activation is mainly regulated by inflammatory responses controlled by NF-κB, AP-1, and ATF-2,[32] we initially examined whether nuclear translocation of these transcription factors is blocked upon HAD-B exposure by measuring their nuclear levels. Interestingly, the extract strongly suppressed the nuclear level of p65, a subunit of NF-κB,[33] but not that of p50, at 15 and 30 min (Fig. 2A), implying that blockade of NF-κB activation is one of the major inhibitory pathways. Furthermore, HAD-B inhibited phosphorylation of ATF-2, but not AP-1 (c-Jun, Fra-1, and c-Fos), indicating that ATF-2 is another target transcription factor. HAD-B-mediated suppression of LPS-induced inflammatory responses with $IC_{50}$ values of 115 (NO) and 156 ($PGE_2$) μg/mL was not strong, but comparable to those of other medicinal plants, such as *Phaseolus calcaratus*, *Sorbus commixta*, *Sanguisorba officinalis*, *Acer tegmentosum*, *Hibiscus cannabinus*, and *Cinnamomum camphora*, with $IC_{50}$ values ranging from 100 to 300 μg/mL.[34–38] These results indicate that the inhibitory activity of HAD-B is derived from its ability to block LPS/TLR4-mediated inflammatory responses at the transcriptional level regulated by NF-κB and ATF-2.

The upstream signaling events for activation of NF-κB and ATF-2 were recently determined. We further analyzed

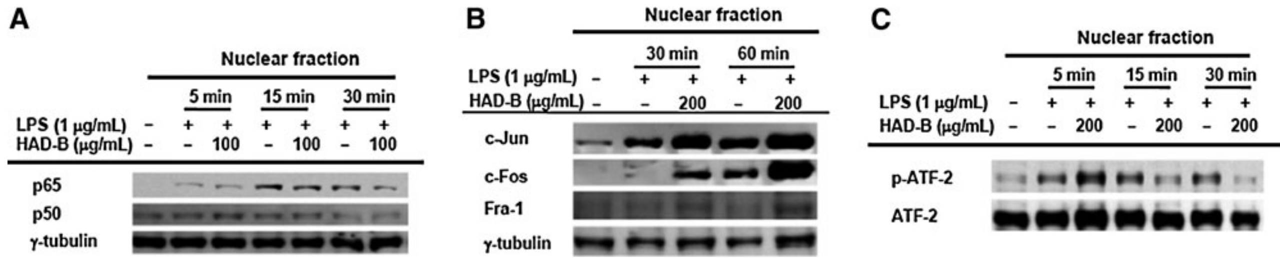


**FIG. 2.** Effects of HAD-B on transcription factor activation. **(A–C)** The protein levels of NF-$\kappa$B, AP-1 family, ATF-2, and $\gamma$-tubulin in nuclear fractions from LPS-activated RAW264.7 cells pretreated with HAD-B (200 $\mu$g/mL) were determined using immunoblot analysis. Levels of phospho- or total p65, p50, c-Jun, c-Fos, ATF-2, and Fra-1 after immunoblotting. NF, nuclear factor; ATF, activating transcription factor; AP, activator protein.

the upstream events using immunoblotting approaches. HAD-B strongly suppressed phosphorylation of I$\kappa$B$\alpha$, a negative regulator of NF-$\kappa$B,[39] which is a critical step in NF-$\kappa$B activation,[40] at 5 min (Fig. 3A). Furthermore, phosphorylation of upstream enzymes responsible for I$\kappa$B$\alpha$ phosphorylation, such as IKK and p85/PI3K, was suppressed by HAD-B at the 5-min time point (Fig. 3A), implying that the real target is located upstream of PI3K. Interestingly, our data strongly suggest that non receptor-type protein tyrosine kinases (Syk and Src) associated with TLRs and their adaptor molecules, such as MyD88 and TRIF,[41] are targets of HAD-B (Fig. 3B). The extract significantly suppressed activation at 2 and 3 min, as assessed from tyrosine phosphorylation levels of the kinases.[42] The issue of whether the inhibition of tyrosine phosphorylation of both Src and Syk by HAD-B is linked to direct suppression of kinase activity was examined with a kinase assay using purified Syk or Src. Intriguingly, HAD-B suppressed the kinase activity of Syk, but not that of Src, suggesting that some of the components in HAD-B act as direct Syk kinase inhibitor(s), while HAD-B-mediated inhibition of Src phosphorylation is indirectly triggered. Recent studies have shown that several ethnomedicinal herbs, such as *C. militaris*, *Kaempferia parviflora*, *S. commixta*, include unidentified compounds that act directly as Syk inhibitors.[36,43] Critical roles of Syk and Src in LPS/TLR4-mediated inflammatory pathways have been established using specific inhibitors, such as BAY61-3606, piceatannol, and PP2. For example, piceatannol is able to block the production of proinflammatory cytokines.[42] PP2 has been also reported to suppress the release of inflammatory mediators, such as NO and $PGE_2$[44] Coincidently, TLR ligand treatment with LPS,

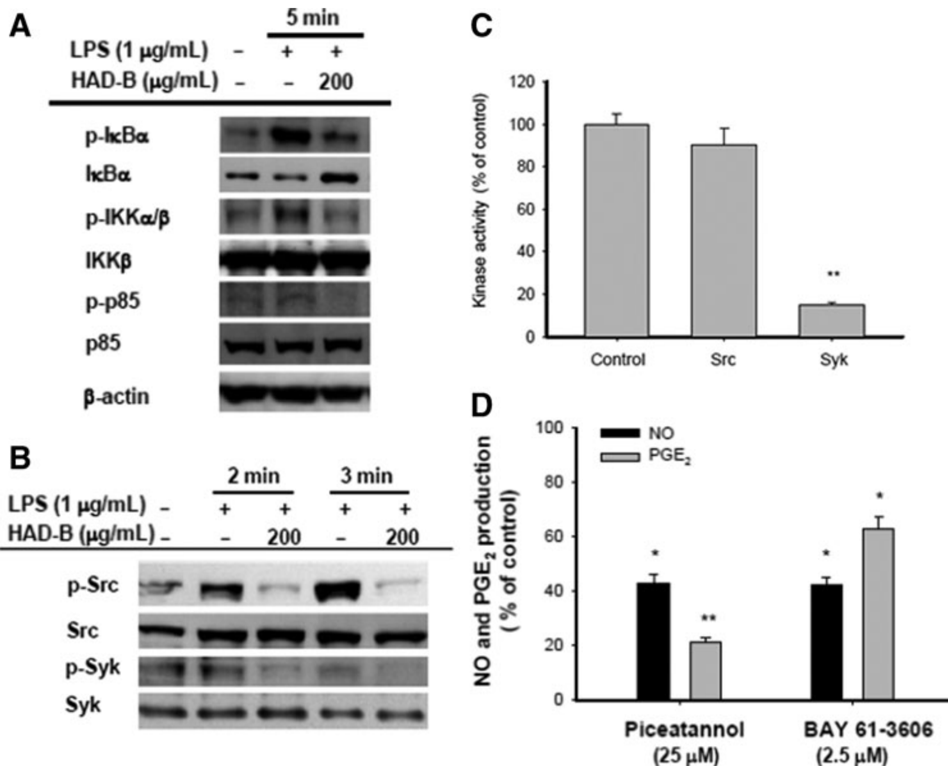


**FIG. 3.** Effects of HAD-B on upstream signaling for NF-$\kappa$B activation. **(A, B)** The phospho- or total protein levels of I$\kappa$B$\alpha$, IKK, p85, Src, Syk, and $\beta$-actin in whole lysates from LPS-activated RAW264.7 cells pretreated with HAD-B (200 $\mu$g/mL) were determined using immunoblot analysis. **(C)** Syk and Src kinase activities were determined with a direct kinase assay using purified enzymes. The control was set as 100% for each enzyme (Src or Syk) as the activity obtained with vehicle-only treatment. **(D)** Inhibitory effects of piceatannol and BAY 61-3606 on NO and $PGE_2$ production were examined with the Griess assay and EIA. *$P < .05$ and **$P < .01$, compared to the control group. Syk, spleen tyrosine kinase; I$\kappa$B$\alpha$, inhibitors of $\kappa$B$\alpha$; IKK, I$\kappa$B$\alpha$ kinase.

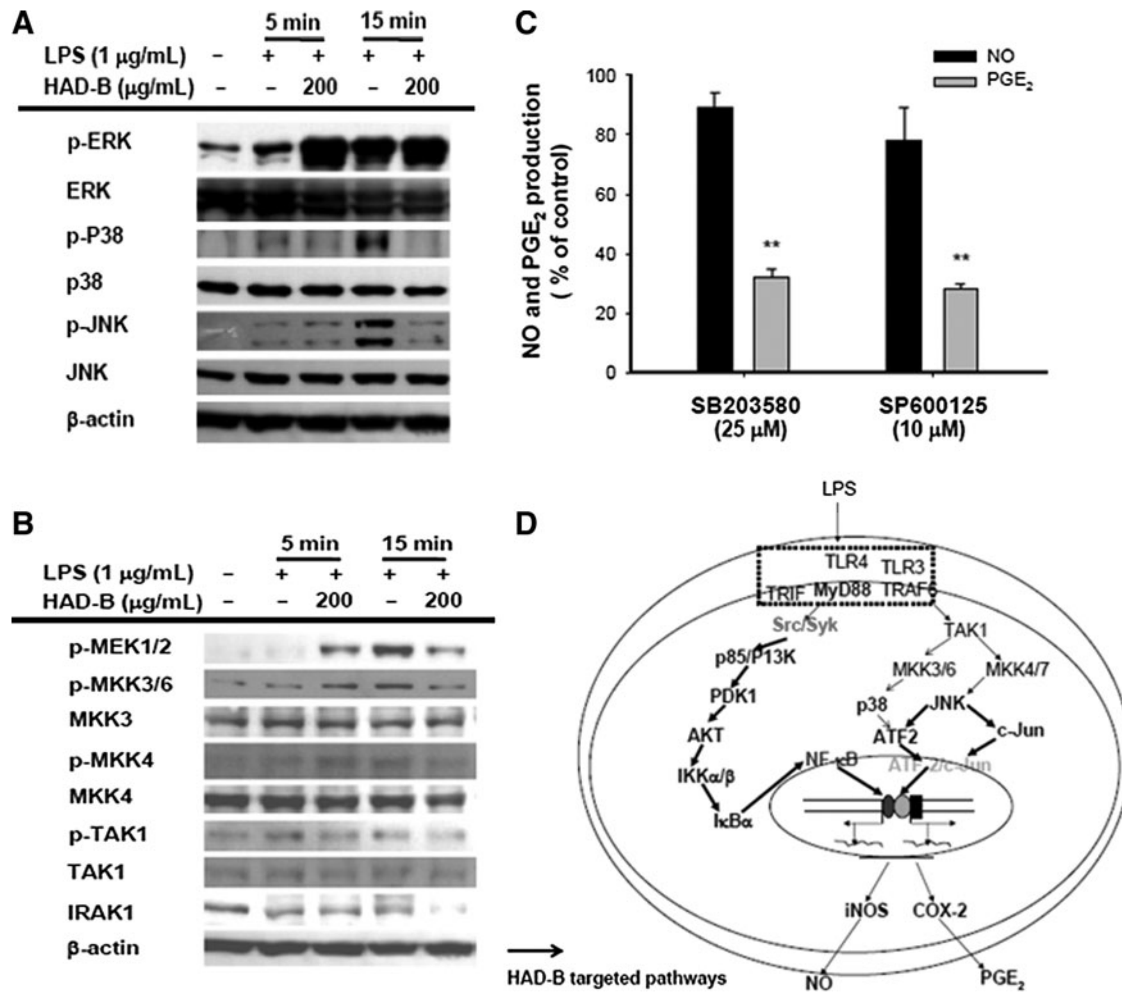


**FIG. 4.** Effects of HAD-B on upstream signaling for ATF-2 activation. **(A, B)** Phospho- or total protein levels of p38, ERK, JNK, MEK1/2, MKK3/6, MKK4, TAK1, IRAK1, and $\beta$-actin in whole lysates from LPS-activated RAW264.7 cells pretreated with HAD-B (200 $\mu$g/mL) were determined using immunoblot analysis. **(C)** Inhibitory effects of SB203580 and SP600125 on NO and $PGE_2$ production were examined with the Griess assay and EIA. **(D)** Schematic diagram of potential inhibitory pathways of HAD-B to modulate macrophage inflammatory responses. $^{**}P < .01$, compared to the control group. ERK, extracellular signal-regulated kinase; MKK, mitogen-activated protein (MAP) kinase kinase; JNK, c-Jun N-terminal kinase; TAK1, transforming growth factor $\beta$–activated kinase 1; IRAK, IL-1 receptor-associated kinase; MEK, MAP ERK kinase.

poly(I:C), and pam3CSK enhances the kinase activity of Syk.[45,46] Moreover, Syk is able to associate with TLR4.[47] Under our conditions, two Syk kinase inhibitors strongly suppressed NO and $PGE_2$ production (Fig. 3D). Furthermore, HAD-B markedly inhibited I$\kappa$B$\alpha$ phosphorylation at 5 min (Fig. 3A), which was predominantly mediated by Syk kinase,[42] strongly supporting a role of Syk as an anti-inflammatory target of HAD-B.

AP-1 and ATF-2 are other critical transcription factors that regulate LPS-mediated inflammatory gene expression.[48,49] Several AP-1 family and CRE-binding proteins translocate to the nucleus during LPS stimulation.[48] However, under our experimental conditions, effective inhibition of AP-1 activation by HAD-B treatment was not observed, rather, the nuclear levels of c-Jun, Fra-1, and c-Fos were enhanced (Fig. 2B). In contrast, HAD-B suppressed phosphorylation of ATF-2 to a significant extent at 15–30 min (Fig. 2C). The upstream signaling enzymes regulating ATF-2 phosphorylation targeted by HAD-B were examined by measuring their phosphorylation levels. Interestingly, phosphorylation levels of p38 and JNK at 5–15 min were dramatically decreased by HAD-B (Fig. 4A), suggesting that these pathways are relevant in the inhibitory mechanism. Conversely, the upregulation of AP-1 seems to be regulated by enhanced levels of ERK and MEK1/2 phosphorylation at 5 min, according to Figure 4. Nevertheless, the biological significance of increased levels of c-Jun, Fra-1, and c-Fos in HAD-B-treated cells was not determined yet. In terms of gene expression modulated by the AP-1 family, however, we will verify the expression levels of such genes in the following experiments. Since the phosphorylation levels of p38 and JNK are promoted by upstream kinases, MKK3/6 and MKK4/7, we further investigated the effects of HAD-B on these enzymes. As shown in Figure 4B, phosphorylation levels of MKK3/6, MKK4/7, as well as TAK1 were clearly inhibited at 5 and 15 min following

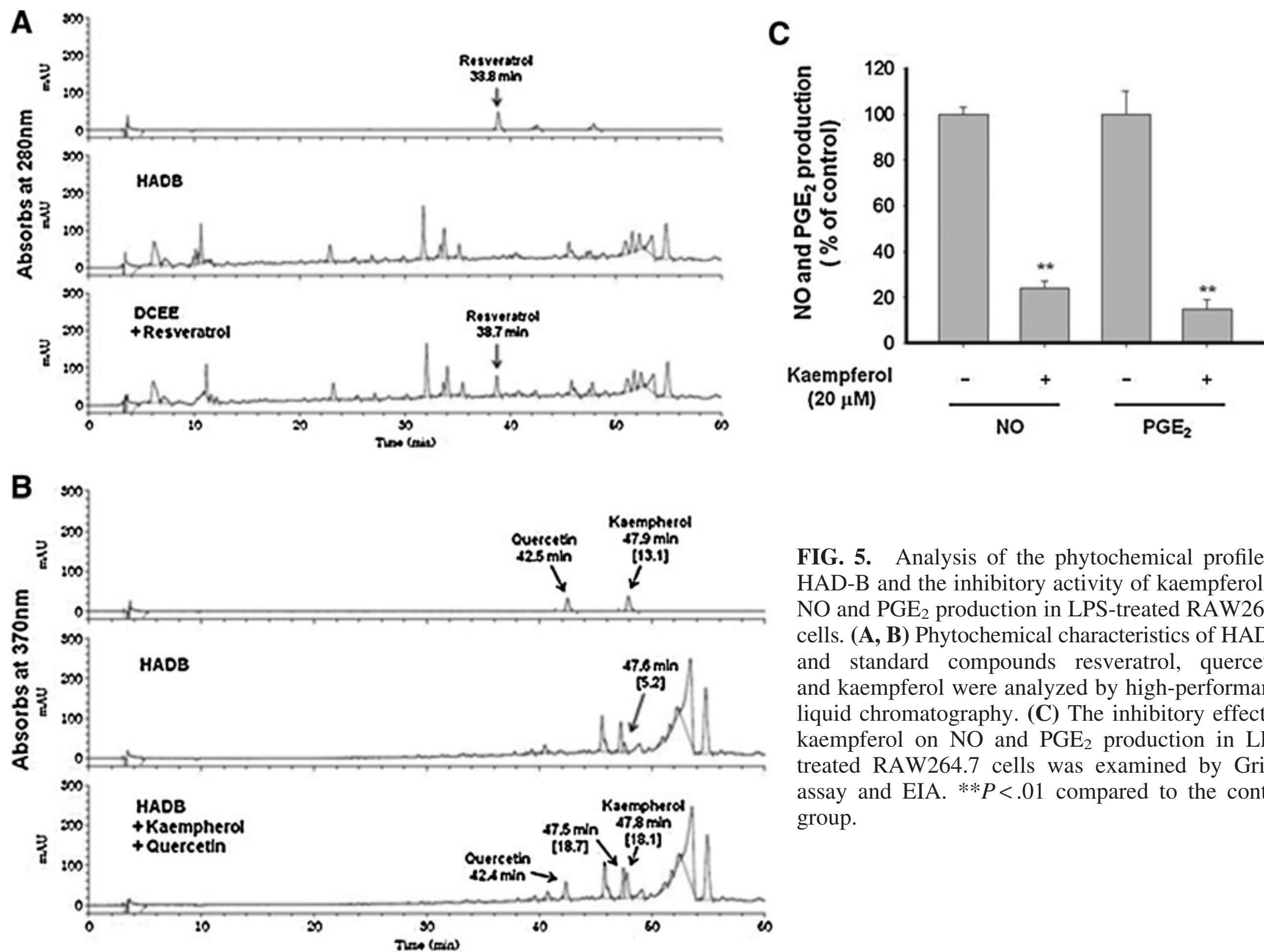


**FIG. 5.** Analysis of the phytochemical profile of HAD-B and the inhibitory activity of kaempferol on NO and $PGE_2$ production in LPS-treated RAW264.7 cells. **(A, B)** Phytochemical characteristics of HAD-B and standard compounds resveratrol, quercetin, and kaempferol were analyzed by high-performance liquid chromatography. **(C)** The inhibitory effect of kaempferol on NO and $PGE_2$ production in LPS-treated RAW264.7 cells was examined by Griess assay and EIA. $^{**}P < .01$ compared to the control group.

HAD-B exposure (Fig. 4B). Degradation of IRAK, signifying activation of the upstream enzyme, TAK1,[50] was not abrogated upon HAD-B exposure, suggesting that IRAK is not a target of HAD-B for the ATF-2 inhibitory route. Meanwhile, according to the inhibitory patterns of SB203580, a p38-specific inhibitor, and SP600125, a JNK inhibitor, we propose that the NO inhibitory effect of HAD-B (Fig. 1A) is not due to suppression of the MAPK/ATF-2 pathway, since NO production was not affected by the inhibitors (Fig. 4C). SB203580 and SP600125 strongly suppressed $PGE_2$ release, whereas NO production was not reduced (Fig. 4C), suggesting that p38 and JNK play critical roles in COX-2 and $PGE_2$ production, as reported previously.[37,42]

Although HAD-B is a mixture of ethnopharmacologically valuable herbal extracts, identification of active components is essential to understand its anti-inflammatory pharmacology. Therefore, we evaluated which components are included in this extract using HPLC analysis with standard compounds, resveratrol, quercetin, and kaempferol, which are known anti-inflammatory compounds.[41,51,52] As seen in Figure 5, kaempferol, but not quercetin and resveratrol was identified from this extract. Based on the level of peak area of standard kaempferol, the content of kaempferol was calculated as 0.001987%. Whether kaempferol is able to suppress the release of NO and $PGE_2$ in LPS-treated RAW264.7 cells was finally investigated using standard kaempferol. As we expected, this compound strongly diminished the production of NO and $PGE_2$ at 20 $\mu$M, suggesting that kaempferol could be the active component acting as a major anti-inflammatory compound in this extract. Nonetheless, the expected content of kaempferol by HPLC analysis of HAD-B seems not to be enough to exert significant inhibitory activity, implying that there are other unidentified active compounds in this extract. Indeed, several peaks with structural similarity to kaempferol were detected between 40 and 50 min (Fig. 5B). Considering that kaempferol displayed strong anti-inflammatory properties (Fig. 5C), and major peaks were similarly seen with kaempferol at 340 nm between 40 to 50 or even 50 to 60 min (Fig. 5C), flavonoid-type compounds could act as major principles contributing to the pharmacological activity of HAD-B. So far, unfortunately, we do not have direct evidence to explain what those peaks are and how strongly these components inhibit inflammatory responses. In terms of these questions, therefore, the answers will be addressed in subsequent experiments.

In 2004, ‘‘The Times’’ highlighted the pathophysiological significance of chronic inflammation by describing it as the ‘‘secret killer.’’ Since then, a number of studies have

demonstrated that inflammation causes a range of serious diseases, such as cancer, vascular disorders, and diabetes.[53,54] Although the molecular mechanisms by which inflammation triggers various diseases are not fully understood at present, toxic radicals and pro- and anti-inflammatory cytokines, such as TNF-$\alpha$, TGF-$\beta$, IL-6, and IL-10, have been identified as critical factors in tumorigenic responses of cancer cells, including invasion, metastasis, migration, angiogenesis, and infiltration.[17,55,56] Recent studies have additionally highlighted the cellular function of tissue-associated macrophages in cancerous conditions. These cells infiltrate tumor tissues and positively manage the release of various cytokines to facilitate tumor cell survival and metastasis.[57] HAD-B has shown efficacy in inhibiting migration and proliferation of human umbilical vein endothelial cells and limiting the formation of capillary tube structures.[13] Furthermore, HAD has been used for the treatment of solid tumors, including pancreatic, lung, colorectal, and stomach cancers, since its development in 1996.[58] Accordingly, it is proposed that the anti-inflammatory activity of HAD-B strongly contributes to its anti-cancer property through functional suppression of tumor-associated macrophages, in addition to its direct anti-cancer activity.

In conclusion, HAD-B inhibits the production of NO and $PGE_2$ at the transcriptional level. Suppression of NF-$\kappa$B activation by both Syk and Src and TAK1/ATF-2 linked to MKK3/6/p38 and MKK4/7/JNK (summarized in Fig. 4D) is a putative inhibitory pathway target of HAD-B action. Since chronic inflammatory conditions contribute to tumorigenic responses and cancer metastasis, the anti-inflammatory activity of HAD-B may additionally be responsible for its anti-cancer activity. The therapeutic efficacy of HAD-B as a potent anti-inflammatory remedy will be further examined using detailed animal arthritis and gastritis models, and the active components identified.

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## AUTHOR DISCLOSURE STATEMENT

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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