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# Anti-inflammatory Effect of the 5,7,4′-Trihydroxy-6-geranylflavanone Isolated from the Fruit of *Artocarpus communis* in S100B-Induced Human Monocytes

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The fruit of *Artocarpus communis* Moraceae, a traditional starch crop, is a rich source of phytochemicals, such as flavonoids and their derivatives. The aim of this study was to investigate whether 5,7,4′-trihydroxy-6-geranylflavanone (AC-GF), a geranyl flavonoid derivative isolated from the fruits of *A. communis*, could decrease the activation of inflammatory mediators induced by S100B (ligand of receptor for advanced glycation end products, RAGE) in THP-1 monocytes. According to the results, low levels of AC-GF ($\leq$2.5 $\mu$M) showed a great inhibitory effect on gene expression of RAGE and down-regulated both TNF-$\alpha$ and IL-1$\beta$ secretion and gene expression ($p < 0.05$). AC-GF also decreased reactive oxygen species (ROS) production in response to S100B ($p < 0.05$). Additionally, Western blotting revealed that AC-GF could effectively attenuate RAGE-dependent signaling, including expression of protein kinase C (PKC) and p47phox, phosphorylation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK), and particularly NF-$\kappa$B activation ($p < 0.05$). In conclusion, this is the first report that AC-GF possesses great antioxidant and anti-inflammatory properties in vitro. This finding may contribute to increased implication and utilization of the fruit of *A. communis* Moraceae in functional foods.

**KEYWORDS: 5,7,4′-Trihydroxy-6-geranylflavanone; RAGE; THP-1; S100B; *Artocarpus communis* Moraceae**

## INTRODUCTION

Inflammation is the first response of the immune system to infection (*1*). Under the circumstances, the injured or infected cells secrete eicosanoids and cytokines, which are pro-inflammatory mediators, producing an inflammatory microenvironment (*2*). Increasing evidence shows that rising pro-inflammatory mediators may promote many lesions in the body, such as malignant tumors (*3*), renal failure (*4*), liver disease (*5*), gastrointestinal disease (*6*), and cardiovascular disease (*7*). Therefore, attenuating an inflammatory response involved in these lesions may be an important issue.

Recent studies suggest that an inflammatory response triggered by the activation of the receptor for advanced glycation end-products (RAGE) plays the decisive role in several diseases such as cancer, ulcer, Alzheimer's disease, and diabetes (*3*, *7*−*9*). RAGE is a receptor with diverse ligands of the immunoglobulin superfamily on cell surfaces (*10*, *11*). Its ligands include products of nonenzymatic glycation, AGEs, members of the S100/calgranulin family, $\beta$-sheet fibrillar structures such as $\beta$-amyloid, and amphoterin (*12*). They can engage RAGE, leading to sustained receptor-dependent signaling and activation of MAPK pathways and NF-$\kappa$B, which in turn evokes an inflammatory response (*13*). S100B, one of the RAGE ligands, is a member of the S100 family of EF-hand $Ca^{2+}$-binding proteins (*14*). It now serves as a valuable tool in the study of RAGE signaling and inflammatory responses (*15*, *16*). Rahbar's group indicated that S100B can amplify monocyte inflammatory responses via RAGE (*17*). Engagement of RAGE with S100B can trigger a series of signalings that result in the production of key inflammatory mediators in endothelial cells, monocytes, phagocytes, and lymphocytes (*14*, *15*, *18*). Taken together, inhibition of S100B−RAGE ligation, as well as the RAGE signaling pathway, may be associated with the prevention and treatment of inflammation-associated diseases.

The genus *Artocarpus* (Moraceae) comprises 50 species, some of which have been investigated phytochemically and biologically (*19*−*21*). *Artocarpus communis* is cultivated in tropical and subtropical regions as a traditional starch crop. Its fruit, also known as breadfruit, has a special flavor of bread after roasting. It can be eaten directly after roasting, baking, and frying and further processed into numerous foods, such as cookies and jelly. Its roots have also been used in traditional medicine as an antiphlogistic, diuretic, and expectorant; additional uses include the treatment of headache, beriberi, vomiting, and dropsy (*22*, *23*). Adewole and Ojewole (*24*) indicated that water extracts of *A. communis* root induced acute hyperglycemia in Wistar rats. Some prenylated flavonoids and derivatives isolated from this plant show interesting biological activities, such as inhibition of nitric oxide production in LPS-stimulated RAW264.7 cells (*25*) and improving arachidonic acid-induced ear

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edema (*19*). Lin and his colleagues also showed that prenylflavonoids isolated from *A. communis* have antiplatelet and anti-inflammatory properties (*26*, *27*). In our previous papers, we isolated five chalcone derivatives from the leaves of *A. communis* that showed anticancer activity (*28*). It was interesting to note that the metabolites in the fruit of *A. communis* may be a variety of bioactive compounds.

In this study, a series of phytochemical examinations on the ethyl acetate layer of the methanol extract of the fruit of *A. communis* was undertaken and led to the isolation and characterization of the geranyl flavonoid derivative 5,7,4′-trihydroxy-6-geranylflavanone (AC-GF). We then determined the structure of this compound and analyzed its effect on the inflammatory response triggered by RAGE activation in S100B-induced human monocytes.

## MATERIALS AND METHODS

**Chemicals.** S100B protein (bovine brain), D-glucose, mannitol, ribose, and RNase were purchased from Sigma Chemical (St. Louis, MO). RPMI-1640 glucose-free medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). L-Glutamine, penicillin−streptomycin solution, and sodium pyruvate solution were obtained from Hyclone (Logan, UT). 6-Carboxy-2′,7′-dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR). Anti-ERK1/2, antiphospho-ERK1/2, anti-p38, antiphospho-p38, anti-PKC, anti-p47phox, anti-NF-$\kappa$B subunit p65, antiphospho-p65, anti-I$\kappa$B$\alpha$, and anti-$\beta$-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). A Trizol RNA isolation kit was obtained from Life Technologies (Rockville, MD); primers for RT-PCR, dNTP, reverse transcriptase, and Taq polymerase were obtained from Gibco BRL (Cergy Pontoise, France). All other chemicals used were of the highest purity available.

**Plant Material.** The fruit of *A. communis* was collected at Tainan Hsien, Taiwan, in July 2006.

**Extraction and Isolation of the Geranyl Flavonoid Derivative from the Fruit of *A. communis*.** The air-dried fruit (2.8 kg) was extracted with methanol (20 L) two times in a percolator and filtered. The filtrate was evaporated by vacuum to give a dark brown residue, which was suspended in water and partitioned with ethyl acetate (EtOAc). The EtOAc-soluble extract (39.2 g) was subjected to chromatography over a silica gel (70−230 mesh, Merck) and eluted with a step gradient of $n$-$C_6H_{14}$/EtOAc solvent system (from 10:1 to pure EtOAc) to give 18 fractions (F1−F18) by TLC profile. Fraction 5 (1.43 g) was chromatographed over a silica gel (230−400 mesh, Merck) with a $C_6H_6$/EtOAc solvent system (from 10:1 to pure EtOAc) to give 10 subfractions (F501−F510). Subfraction F506, eluted with $C_6H_6$/EtOAc (7:1), was further purified by preparative TLC developing with $CH_2Cl_2$/EtOAc (8:1) to obtain the geranyl flavonoid derivative (AC-GF, 11.2 mg).

**Spectrometry.** Optical rotations were measured with a JASCO DIP-370 digital polarimeter (JASCO Ltd., Tokyo, Japan). UV spectra were obtained on a Thermo model $\alpha$ UV−vis spectrophotometer (Thermo Spectronic, Cambridge, U.K.). IR spectra were recorded on a Perkin-Elmer 2000 FT-IR spectrophotometer (Fremont, CA). $^1$H (400 MHz) and $^{13}$C NMR (100 MHz) experiments were performed with a Varian Unity 400 NMR spectrophotometer (Varian, Palo Alto, CA). ESI/MS measurements were performed on a Bruker Daltonics APEX II 30e mass spectrometer (Bruker Daltonics, Billerica, MA) .

Compound AC-GF was named 5,7,4′-trihydroxy-6-geranylflavanone. Its chemical and physical properties were as follows: pale yellow powder; $[\alpha]_D$ −8.2° ($CHCl_3$, $c$ 0.4); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$), 228 (4.42), 292 (4.31), 334 (3.70); IR (KBr) $\upsilon_{max}$ cm$^{-1}$, 3310, 1638, 1588, 1524; $^1$H NMR (400 MHz, $CDCl_3$), $\delta$ 1.58, 1.66, and 1.70 (each 3H, s), 2.02 and 2.07 (each 2H, m), 2.80 (1H, dd, $J$ = 17.2, 3.2 Hz), 3.05 (1H, dd, $J$ = 17.2, 12.8 Hz), 3.31 (2H, d, $J$ = 6.8 Hz), 5.04 (1H, t, $J$ = 7.5 Hz), 5.20 (1H, t, $J$ = 7.6 Hz), 5.34 (1H, dd, $J$ = 12.8, 3.2 Hz), 6.03 (1H, s), 6.60 (1H, br s, OH), 6.88 (2H, d, $J$ = 8.8 Hz), 7.31 (2H, d, $J$ = 8.8 Hz), 12.01 (1H, s, OH); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 196.7 (s), 164.1 (s), 162.0 (s), 159.8 (s), 156.0 (s), 138.4 (s), 131.9 (s), 130.6 (s), 127.7 × 2 (d), 123.8 (d), 121.4 (d), 115.6 × 2 (d), 106.5 (s), 103.0 (s), 96.8 (d), 78.7 (d), 42.9 (t), 39.7 (t), 26.3 (t), 25.6 (q), 21.7 (t), 17.7 (q), 16.1 (q); ESI/MS $m/z$ $[M + Na]^+$ 431.

**Cell Culture and Treatments.** The human THP-1 monocytic cell line (THP-1) was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in RPMI-1640 medium, supplemented with 10% FBS, glutamine (2 mM), HEPES (10 mM), streptomycin−penicillin (100 mg/mL/100 U/mL), 50 $\mu$M $\beta$-mercaptoethanol, and 5.5 mM D-glucose (normal glucose; NG) at 37 °C, in a humidified atmosphere of 95% air and 5% $CO_2$. For inflammatory conditions, cells were cocultured with 6.5 $\mu$g/mL S100B protein and treated with AC-GF as indicated. In our preliminary test, the cell viability of THP-1 cells was tested using the MTT assay and was not significantly affected when incubated with 6.5 $\mu$g/mL S100B protein and AC-GF up to a concentration of 2.5 $\mu$M.

**RNA Preparation and RT-PCR.** Total RNA was isolated from human monocytic THP-1 cells ($2 \times 10^6$ cells/mL) after treatment with 6.5 $\mu$g/mL S100B and 1 or 2.5 $\mu$M AC-GF for 4 h by Trizol RNA isolation kit (Rockville, MD) as described in the manufacturer's manual. The primer sequences used are the following: TNF-$\alpha$ (forward 5′-CCAAACGATG-TTGTACCCGA-39, reverse 5′-CAGTTGGAGGAGAGACGGTA-3′); IL-1$\beta$ (forward 5′-CTCTCTCACCTCTCCTACTCAC-3′, reverse 5′-ACA-CTGCTACTTCTTGCCCC-3′); RAGE (forward 5′-AGAGGAGAGGA-AGGCCCCAGA-3′, reverse 5′-GGCAAGGTGGGGTTATACAGG-3′), and 18S (forward 5′-TTGGAGGGCAAGTCTGGTG-3′, reverse 5′-CC-GCTCCCAAGATCCAACTA-3′). Briefly, from each sample, cDNA corresponding to 0.05 $\mu$g of RNA was reverse-transcribed, using 200 U of Superscript II reverse transcriptase, 20 U of RNase inhibitor, 0.6 mM dNTP, and 0.5 $\mu$g/mL of oligo(dT). PCR analyses were performed on the aliquots of the cDNA preparations to detect TNF-$\alpha$, IL-1$\beta$, RAGE, and 18S (as an internal standard) gene expression using the FailSafe PCR system (Epicenter Technologies, Madison, WI). The reactions were performed in a volume of 50 $\mu$L containing (final concentrations) 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MnCl_2$, 0.2 mM dNTP, 2 U of Taq DNA polymerase, and 50 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95 °C, 29−35 cycles of amplification (the annealing temperature for TNF-$\alpha$ and 18S was 59 °C and that for IL-1$\beta$ and RAGE was 56 °C) were performed, followed by a 10 min final extension at 72 °C.

**Analysis of PCR Products.** PCR products were fractionated on 1.8% agarose gels containing 1× SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA). The gel was then photographed under UV transillumination. For quantification, the PCR bands on the photograph of the gel were scanned using a densitometer, linked to a computer analysis system, LabWorks 4.5 (Upland, CA). The results were expressed as fold stimulation over control group after the gene signal had been normalized relative to the corresponding 18S signal from each sample.

**Western Blotting.** The protein fractions were isolated from human monocytic THP-1 cells ($2 \times 10^6$ cells/mL) after treatment with 6.5 $\mu$g/mL S100B and 1 or 2.5 $\mu$M AC-GF for 4 h. Briefly, the total proteins were extracted by the Total Protein Extraction Kit (Millipore, Bedford, MA) following the manufacturer's instruction. Protein concentration was measured by Bradford assay with BSA as a standard. Total protein and compartmental protein extracts (20−50 $\mu$g of protein) were separated on 8% SDS−polyacrylamide minigels for PKC and p47phox detection and on 12% SDS−polyacrylamide minigels for MAPK family (p38 MAPK and ERK1/2) protein detection and then transferred to PVDF membranes (Millipore) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membrane was blocked in StartingBlock Blocking Buffers (Pierce, Rockford, IL) for 15 min at room temperature and then incubated overnight at 4 °C with indicated primary antibodies (1:1000 dilutions). After hybridization with primary antibodies, the membrane was washed with Tris-buffered saline containing Tween-20 (TBST) three times, incubated with HRP-labeled secondary antibody for 45 min at room temperature, and washed with TBST three times. Final detection was performed with enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The relative protein expression was quantified densitometrically using LabWorks 4.5 software (Cambridge, U.K.) and calculated according to the reference bands of the loading control.

**Cyotokine ELISA Assays.** THP-1 cells were incubated in six-well tissue culture plates in RPMI-1640 medium and cotreated with S100B and 1 or 2.5 $\mu$M AC-GF for 12 h. The supernatant-conditioned medium was then harvested and assayed for TNF-$\alpha$ and IL-1$\beta$ secretion using a specific ELISA kit according to the manufacturer's instructions (Pierce Endogen,

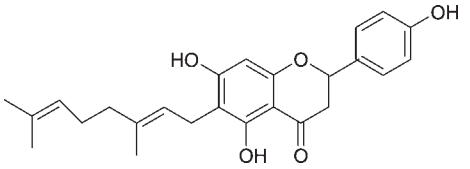


**Figure 1.** Chemical structure of AC-GF isolated from the fruit of *Artocarpus communis* Moraceae.

Rockford, IL). The cells incubated under the conditions without S100B and 1 or 2.5 $\mu$M AC-GF for 12 h were used as a blank control for the ELISA.

**Intracellular ROS Production Assay.** Intracellular ROS generation was detected using the fluorescent probe DCF-DA. DCF-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity is believed to parallel the amount of ROS formed intracellularly. THP-1 cells were incubated with 6.5 $\mu$g/mL S100B and 1 or 2.5 $\mu$M AC-GF for 4 h. At the end of incubation, cells ($2 \times 10^6$ cells/mL) were collected and resuspended with PBS and then stained with DCF-DA (20 $\mu$M) for 15 min at 37 °C. After incubation, cells were resuspended in ice-cold PBS and then transferred to a 12-well plate for examination of cell morphology. The results were observed by IX71 microscope (Olympus, Osaka, Japan) and recorded by ImagePro (Media Cybernetics, Bethesda, MD). In addition, an aliquot of the suspension (200 $\mu$L) for each treament was loaded into a 96-well plate for measuring DCF fluorescence. The DCF fluorescence intensity was detected using a FLUOstar galaxy fluorescence plate reader (BMG Labtechnologies, Offenburg, Germany) with an excitation wavelength at 485 nm and an emission wavelength at 530 nm. After detection of fluorescence intensity, the total protein of cells was measured by Bradford assay for internal control in each well.

**Statistical Analysis.** Each experiment was performed in triplicate. The results are expressed as means ± SD. Statistical comparisons are made by a one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. Differences are considered to be significant when the $p$ values are $< 0.05$.

## RESULTS

**Isolation and Identification of Geranyl Flavonoid Derivative from the Fruit of *A. communis*.** AC-GF was identified in the EtOAc-soluble fraction of the fruit of *A. communis* and obtained as a pale yellow powder. The $^1$H NMR spectrum of AC-GF revealed the presence of one methylene α to the carbonyl at $\delta$ 3.05 (dd, $J$ = 17.2, 12.8 Hz, $H_{ax}$-3) and $\delta$ 2.80 (dd, $J$ = 17.2, 3.2 Hz, $H_{eq}$-3) and one oxymethine at $\delta$ 5.34 (dd, $J$ = 12.8, 3.2 Hz, H-2). These data suggested that AC-GF possesses a flavanone skeleton. Furthermore, the $^1$H NMR spectrum exhibited the presence of a geranyl group, an $A_2B_2$ type aromatic proton signal in the B ring, a singlet proton at $\delta$ 6.03, and the low-field signal at $\delta$ 12.01 (1H, s) indicating a C-5-hydroxy proton intramolecularly hydrogen bonded to the carbonyl oxygen atom. The $^{13}$C NMR spectrum of AC-GF showed 25 carbon signals. The ESI/MS of AC-GF showed a $[M + Na]^+$ at $m/z$ 431. From the above results, a formula was proposed for the structure of AC-GF (**Figure 1**). The chemical shifts of this compound were matched to reported values (*29*).

**Inhibitory Effect of AC-GF on the Expression of Pro-inflammatory Cytokines.** Cytokines, such as TNF-α and IL-1$\beta$, are believed to play an important role in the inflammation triggered by RAGE activation (*3*). In this study, we evaluated the effect of AC-GF on the expression of pro-inflammatory cytokines induced by S100B in THP-1 cells. As shown in **Figure 2**, S100B treatment of THP-1 cells for 4 h led to significantly increased expression of TNF-α and IL-1$\beta$ mRNA ($p < 0.05$). However, significant inhibition of TNF-α mRNA expression was observed with the treatment with 1 and 2.5 $\mu$M AC-GF ($p < 0.05$). In addition, the treatment with

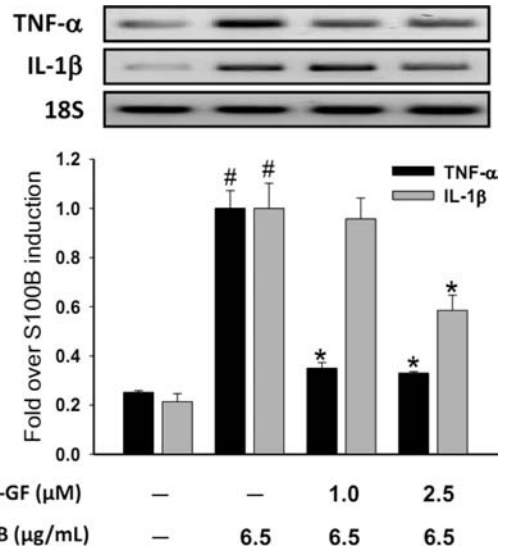


**Figure 2.** Effect of AC-GF on mRNA expression of TNF-α and IL-1$\beta$ in THP-1 cells induced by S100B. Cells were cultured with S100B protein (6.5 $\mu$g/mL) in the absence or presence of AC-GF (1 and 2.5 $\mu$M) for 4 h. mRNA was detected by the RT-PCR method. PCR products were visualized by SyBr-Green staining on 1.8% agarose gel. The basal IL-1$\beta$ and TNF-α mRNA levels of THP-1 cells cultured under S100B conditions at 4 h were set to 1.0, and the relative changes in IL-1$\beta$ and TNF-α mRNA expression were expressed as multiples of data. The upper panel indicates an original band; the lower panel shows the results of densitometric analyses. Results shown are representative of three independent experiments. #, $p < 0.05$ versus vehicle control; ∗, $p < 0.05$ versus S100B treatment only.

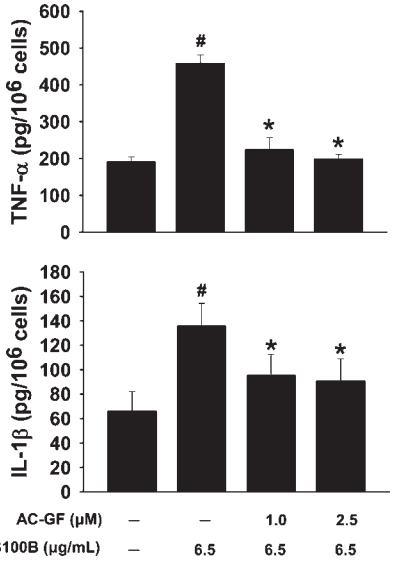


**Figure 3.** Effect of AC-GF on TNF-α and IL-1$\beta$ secreted by S100B-stimulated THP-1 cells. Cells were cultured with S100B protein (6.5 $\mu$g/mL) in the absence or presence of AC-GF (1 and 2.5 $\mu$M) for 12 h. After the incubation period, TNF-α and IL-1$\beta$ in the conditioned medium were detected by ELISA. Results shown are the mean ± SD from three independent experiments. #, $p < 0.05$ versus vehicle control; ∗, $p < 0.05$ versus S100B treatment only.

2.5 $\mu$M AC-GF significantly decreased the mRNA expression of IL-1$\beta$ ($p < 0.05$). The effects of AC-GF on the induction of protein levels of TNF-α and IL-1$\beta$ pro-inflammatory cytokine stimulated by S100B were further determined. **Figure 3** shows similar results by RT-PCR analyses, where AC-GF exerted great

inhibitory effect on both TNF-α and IL-1β secretion in THP-1 cells ($p < 0.05$) cotreated with S100B for 12 h.

**Effect of AC-GF on Intercellular ROS Generation.** Activation of RAGE has been reported to alter the redox status of cells through the overproduction of ROS, leading to inflammation (*3*). The role of ROS as second messengers for inducing expression of various inflammatory genes in monocytes has been reported recently (*17*). In this study, we determined whether S100B induced a pro-oxidant environment in THP-1 cells and estimated the inhibitory effect of AC-GF on ROS production. As shown in **Figure 4**, treatment with S100B in THP-1 cells after 4 h induced an increase of ROS indicated by DCF fluorescence ($p < 0.05$).

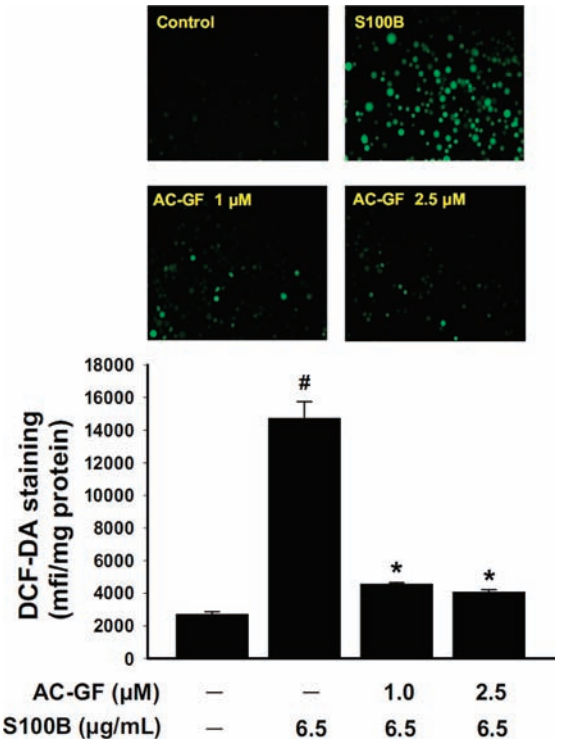


**Figure 4.** Inhibitory effect of AC-GF on ROS generation in THP-1 cells induced by S100B. Cells were cultured with S100B protein (6.5 μg/mL) in the absence or presence of AC-GF (1 and 2.5 μM) for 4 h. The green fluorescence resulting from ROS generation was detected using an IX71 microscope. ROS production was quantified using mean fluorescent intensities with the fluorescent probe DCF-DA as described under Materials and Methods. #, $p < 0.05$ versus vehicle control; ∗, $p < 0.05$ versus S100B treatment only. mfi, mean fluorescent intensities.

However, a significant reduction of ROS was observed after the treatment with AC-GF at a low concentration ($p < 0.05$).

**Effect of AC-GF on Oxidative Stress-Sensitive Protein Expression.** Increased oxidative stress has been reported both in vitro and in vivo in the inflammatory state via production of ROS (*12*). We further investigated the effect of AC-GF on oxidative stress-sensitive protein expression, including p47phox and PKC protein. As shown in **Figure 5**, THP-1 cells treated with S100B significantly elicited the protein expression of p47phox and PKC ($p < 0.05$), whereas AC-GF showed significant inhibition of PKC and p47phox protein expression in S100B-induced THP-1 cells.

**Effect of AC-GF on S100B-Induced Phosphorylation of p38 MAPK and ERK.** Inflammation is related to specifically activated MAPK pathways in human monocytes (*30*). In addition, the production of inflammatory cytokines, such as TNF-α, by activated human monocytes was regulated by the MAPK pathway (*30*). Thus, the effects of AC-GF on protein expression in MAPK signaling pathways activated by S100B were further investigated. As shown in **Figure 6**, THP-1 cells elicited the activation of p38 MAPK and ERK after 4 h of S100B stimulation ($p < 0.05$). The expression of these MAPKs was confirmed with antibodies specific for phospho-p38 and phospho-ERK (**Figure 6**). These results indicated that AC-GF showed significant inhibition of S100B-mediated ERK and p38 MAPK activation in THP-1 cells ($p < 0.05$).

**Effect of AC-GF on the S100B-Induced p65 Subunit of NF-κB Phosphorylation and IκBα Degradation.** S100B-induced inflammatory cytokine gene expression was found to involve the IκB/NF-κB pathway (*14*). The effect of AC-GF on S100B-induced NF-κB activation was examined. Incubation of THP-1 cells with S100B 6.5 μg/mL led to a rapid loss of IκBα and increased the phosphorylation of the subunit p65 of NF-κB (**Figure 7**). However, AC-GF stabilized IκBα degradation induced by S100B to different extents. Similarly, phosphorylation of subunit p65 of NF-κB was inhibited by the treatment of AC-GF in S100B-induced THP-1 cells (**Figure 7**). This phenomenon was consistent with the inhibition of downstream inflammatory factors (TNF-α and IL-1β). These data suggested that the anti-inflammatory effect of AC-GF on activated monocytes could be due to their modulation of NF-κB.

**Effect of AC-GF on Expression of RAGE.** Studies have shown that S100B binding to membrane RAGE can lead to activation of multiple signal transduction events, inflammatory gene expression, and ROS overproduction (*3*, *12*). In the present study, THP-1 cells cultured under S100B conditions for 4 h showed approximately a 5-fold increase in mRNA expression of RAGE as compared to the control group. Intervention of 2.5 μM AC-GF also could attenuate mRNA expression of RAGE (**Figure 8**).

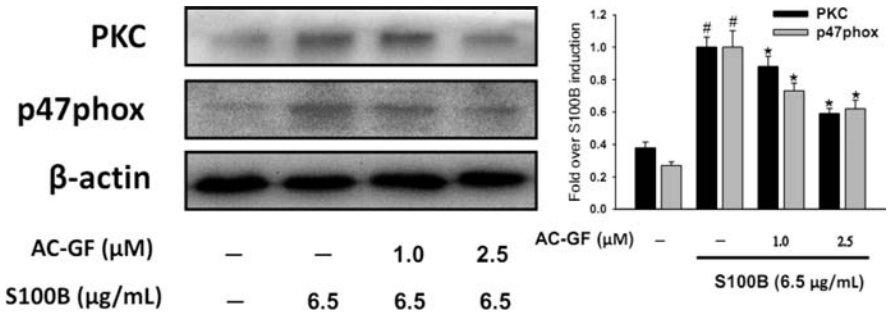


**Figure 5.** Effect of AC-GF on protein expression of PKC and p47phox in THP-1 cells induced by S100B. Cells were cultured in S100B protein (6.5 μg/mL) in the absence or presence of AC-GF (1 and 2.5 μM) for 4 h. Total cell lysates of THP-1 cells were prepared after treatment. Proteins separated by SDS-PAGE were immunoblotted and probed with antibodies of p47phox, PKC, and β-actin. The left panel indicates an original band; the right panel shows the results of densitometric analyses. Results shown are representative of three independent experiments. #, $p < 0.05$ versus vehicle control; ∗, $p < 0.05$ versus S100B treatment only.

## DISCUSSION

There are abundant bioactive compounds, such as flavonoids, in *A. communis* (*24*−*28*). In the past, these compounds were thought to act as antioxidants. However, growing evidence indicates that flavonoids can function in other ways to exhibit positive effects on health (*31*). Although flavonoids have poor bioavailability (*32*), they can protect against cardiovascular disease (*33*) by reducing inflammation (*34*) and improving endothelial function (*35*). Therefore, we hypothesized that the geranyl flavonoid derivative AC-GF (**Figure 1**), of which little was known about its bioactivity, isolated from the fruit of *A. communis* may have anti-inflammatory effects. Apart from *A. communis*, AC-GF was also found in *Mimulus clevelandii* (*36*), *Mimulus lupulus* L. (*37*), *Macaranga alnifolia* (*38*), and *Macaranga pleiostemona* (*39*). In our previous study, we demonstrated that naturally occurring flavonoids such as catechin and quercetin could inhibit the diabetic inflammatory condition by decreasing the following molecules: pro-inflammatory cytokines, TNF-α and IL-1$\beta$; chemokines, MCP-1 and IP-10; adhesion factor, PECAM-1; $\beta_2$-integrin; and COX-2 expression induced by S100B in THP-1 monocytes (*16*). In the same inflammatory condition, we first found that AC-GF showed great inhibitory effects on the key pro-inflammatory mediators, TNF-α and IL-1$\beta$, in activated monocytes (**Figures 2** and **3**). Studies show that overproduction of ROS may contribute to activation of nuclear factor-$\kappa$B (NF-$\kappa$B) (*14*, *17*). This is an important event in the inflammatory condition induced by S100B. As shown in **Figure 4**, we also found that AC-GF could decrease ROS production in response to S100B. Interestingly, the concentration of AC-GF was lower than that of catechin, which was investigated before (*16*), in the treatment for attenuating ROS production in S100B-induced monocytes. Many studies have indicated that NADPH oxidase activated via protein kinase C (PKC) by translocation of p47phox to membranes is the key event of intracellular ROS formation (*30*, *40*). Previously, we found that S100B could induce the expression of oxidative stress-sensitive

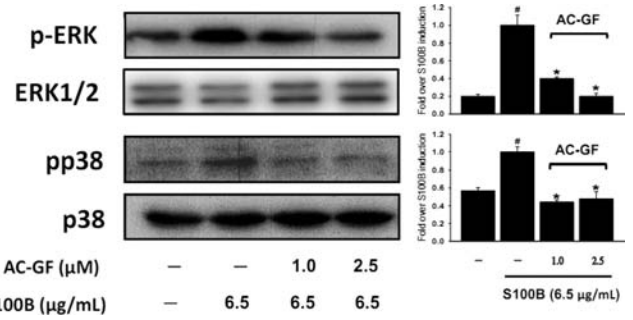


**Figure 6.** Effect of AC-GF on MAPKs phosphorylation in THP-1 cells induced by S100B. Cells were cultured with S100B protein (6.5 $\mu$g/mL) in the absence or presence of AC-GF (1 and 2.5 $\mu$M) for 4 h. After treatment, whole cell lysates were subjected to Western blot analysis using phosphospecific antibodies to p38 MAPK or ERK, then stripped and reprobed with their nonphosphospecific antibodies to show equal loading. The left panel indicates an original band; the right panel shows the results of densitometric analyses. Results shown are representative of three independent experiments. #, $p < 0.05$ versus vehicle control; ∗, $p < 0.05$ versus S100B treatment only.

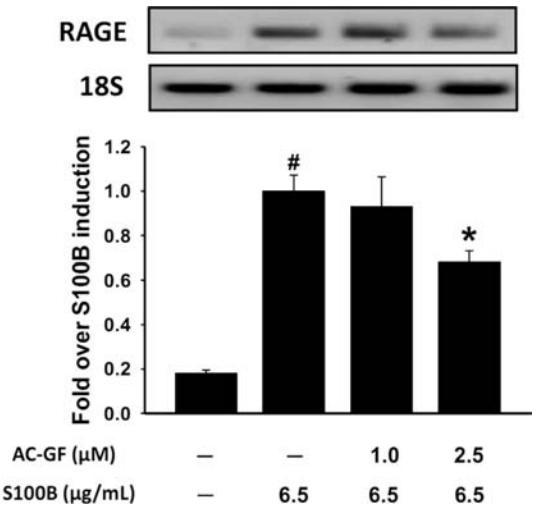


**Figure 8.** Effect of AC-GF on mRNA expression of RAGE in THP-1 cells induced by S100B. Cells were cultured with S100B protein (6.5 $\mu$g/mL) in the absence or presence of AC-GF (1 and 2.5 $\mu$M) for 4 h. mRNA was detected by the RT-PCR method. PCR products were visualized by SyBr-Green staining on 1.8% agarose gel. The basal RAGE mRNA level of THP-1 cells cultured under S100B condition at 4 h was set to 1.0, and the relative changes in RAGE mRNA expression were expressed as multiples of data. The upper panel indicates an original band; the lower panel shows the results of densitometric analyses. Results shown are representative of three independent experiments. #, $p < 0.05$ versus vehicle control; ∗, $p < 0.05$ versus S100B treatment only.

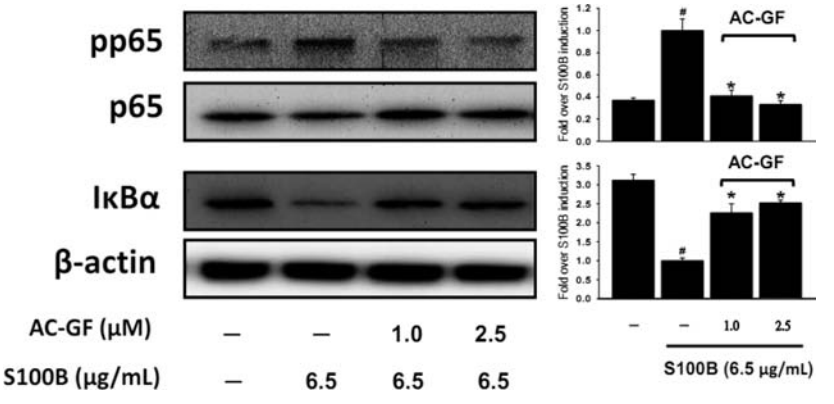


**Figure 7.** Effect of AC-GF on S100B-induced NF-$\kappa$B subunit p65 phosphorylation and I$\kappa$B$\alpha$ protein degradation in THP-1 cells. Cells were cultured with S100B protein (6.5 $\mu$g/mL) in the absence or presence of AC-GF (1 and 2.5 $\mu$M) for 4 h. Total cell lysates of THP-1 cells were prepared after treatment. Proteins separated by SDS-PAGE were immunoblotted and probed with antibodies of phosphorylated p65, p65, I$\kappa$B$\alpha$, and $\beta$-actin. The left panel indicates an original band; the right panel shows the results of densitometric analyses. Results shown are representative of three independent experiments. #, $p < 0.05$ versus vehicle control; ∗, $p < 0.05$ versus S100B treatment only.

**Table 1.** Overview of Effect of AC-GF on THP-1 Monocyte Physiology under S100B-Treated Condition[a]

| | inflammation-associated response involved in S100B-induced THP-1 monocytes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pro-inflammatory cytokines | | | | | | | | | NF-κB-related signaling |
| | | TNF-α | | IL-1β | | oxidative stress-sensitive signaling | | | MAPKs | | |
| group | RAGE mRNA expression | mRNA | protein | mRNA | protein | ROS | PKC | p47phox | p-p38 | p-ERK | IκBα/p65 |
| AC-GF (2.5 μM) | + | +++ | +++ | ++ | ++ | +++ | ++ | ++ | +++ | +++ | +++ |

[a] Inhibition is shown as +/++/+++.

proteins including PKC and p47phox and then lead to ROS burst in human monocytes (*16*). As shown in **Figure 5**, S100B-induced protein expression of PKC and p47phox was attenuated by AC-GF. This may partially prove the anti-inflammatory effect of AC-GF. Taken together, we could conclude that AC-GF may exert good antioxidant and anti-inflammatory activity in S100B-induced human monocytes.

It has been reported that flavonoids may regulate diabetic inflammatory responses via the modulation of oxidative stress through oxidative stress-sensitive and mitogen-activated protein kinase (MAPK) signaling pathways (*16*, *41*). MAPK signaling cascades are stimulated by many extracellular stimuli, such as growth factors, cytokines, and various environmental stresses, and they serve as a common signal transduction pathway for signals involved in proliferation, differentiation, functional activation, and inflammatory responses (*42*). Many studies have demonstrated that ligation with RAGE can induce the phosphorylation of several MAPKs, including extracellular signal-regulated kinase (ERK) and p38 MAPK (*3*), and then contribute to the NF-κB activation and cytokines release (*14*). To clarify the anti-inflammatory mechanism of AC-GF in S100B-induced human monocytes, we further investigated the effect of AC-GF on the RAGE-dependent signaling pathway. The results indicated that AC-GF strongly decreased the phosphorylation of ERK and p38 MAPK (**Figure 6**). These findings are in agreement with TNF-α and IL-1β down-regulation. This suggests that AC-GF may decrease TNF-α and IL-1β secretion via modulation of MAPK signaling in human monocytes.

NF-κB plays a final deciding role in regulating inflammatory genes (*30*). S100B can promote IκBα phosphorylation through a series of signaling events, facilitating the translocation of NF-κB subunit p65 into the nucleus and stimulating the expression of inflammatory factors (*14*). We also found that AC-GF could prevent IκBα degradation and NF-κB subunit p65 phosphorylation (**Figure 7**). This seemed to correlate with the anti-inflammatory characteristics of flavonoids, such as luteolin, rutin, EGCG, quercetin, and catechin (*30*). The regulation of these flavonoids for upstream signaling such as ROS, PKC/p47phox, p38 MAPK, and ERK1/2 did indeed influence downstream NF-κB activation and inhibited the expression of pro-inflammatory cytokine genes (*30*).

RAGE, which exists in vascular cells (monocytes/macrophages, smooth muscle cells, and endothelial cells), can trigger inflammation-associated signal transduction in response to S100B (*7*, *15*). It is not expressed under normal physiological conditions; however, it is overexpressed when its ligands, such as S100B, exist or when transcription factors regulating RAGE are activated (*3*, *6*, *8*, *10*). According to the results shown in **Figure 8**, S100B significantly increased RAGE mRNA expression in THP-1 cells. However, AC-GF could decrease RAGE mRNA expression effectively (**Figure 8**). It seemed that the inhibitory effect of geranyl flavonoid derivative, AC-GF, on RAGE gene expression may be closely related to its anti-inflammatory characteristics.

In conclusion, monocytes are usually involved in the inflammatory response and clearly play key roles in a wide variety of inflammatory diseases (*17*). The present study showed that RAGE ligation with S100B could trigger a strong inflammatory response in human monocytes. As summarized in **Table 1**, our results are the first finding that treatment with AC-GF, the geranyl flavonoid derivative isolated from the fruit of *A. communis*, could attenuate S100B-induced inflammatory response by decreasing ROS and pro-inflammatory cytokines (TNF-α and IL-1β) and inhibiting the RAGE-dependent signalings including oxidative stress-sensitive and NF-κB-related signaling pathways. Moreover, AC-GF possesses potent inhibition of RAGE activation in human monocytes. Taken together, AC-GF possesses great anti-inflammatory properties in vitro. This finding may contribute to increased utilization of the fruit of *A. communis* in health foods.

## ABBREVIATIONS USED

AC-GF, 5,7,4′-trihydroxy-6-geranylflavanone; THP-1 cells, human THP-1 monocytic cells; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; PKC, protein kinase C; RAGE, receptor for advanced glycation endproducts; DCF-DA, 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; S100B, S100B protein; TBST, tris buffered saline containing Tween-20; RT-PCR, reverse transcription polymerase chain reaction; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence.

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