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Anti-inflammatory Activity of 3,6,3′-Trihydroxyflavone in Mouse Macrophages, *In vitro*

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Numerous studies have examined the role of flavonoids in modulating inflammatory responses *in vitro*. In this study, we found a novel flavonoid, 3,6,3′-trihydroxyflavone (**1**), with anti-inflammatory effects. Anti-inflammatory activity and mechanism of action were examined in mouse macrophages stimulated with lipopolysaccharide (LPS). Our results showed that the anti-inflammatory effects of **1** are mediated *via* p38 mitogen-activated protein kinase (p38 MAPK), Jun-N terminal kinase (JNK), and the extracellular-signal-regulated kinase (ERK) pathway in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Binding studies revealed that **1** had a high binding affinity to JNK1 ($1.568 \times 10^8\ M^{-1}$) and that the 3- and 6-hydroxyl groups of the C-ring and A-ring of **1** participated in hydrogen bonding interactions with the side chains of Asn114 and Lys55, respectively. The oxygen at the 3′ position of the B-ring formed a hydrogen bond with side chain of Met111. Therefore, **1** could be a potential inhibitor of JNKs, with potent anti-inflammatory activity.

Key Words : Anti-inflammatory activity, 3,6,3'-Trihydroxyflavone, Flavonoid, JNK, Binding model

Introduction

Inflammation is an early protective response of vascular tissues to tissue injury and is part of the body's immune response. There is also substantial evidence that inflammation is an important constituent of cancer progression, and numerous studies have shown that many cancers occur at the sites of infection and chronic inflammation. *Helicobacter pylori* infection is known to cause gastric cancer and chronic hepatitis and is also thought to cause hepatoma.[1,2] Many studies have shown that nonsteroidal anti-inflammatory drugs, targeting cyclooxygenase (COX)-2, including aspirin, reduce cancer mortality rates in several cancer types.[3] The relationship between epithelial cancer cells and stromal cells, including fibroblasts and endothelial cells, is known to play a major role in cancer growth and progression through the recruitment of inflammatory cells.[4] When cancer-related fibroblasts and macrophages are stimulated by epithelial cancer cells, many cytokines and chemokines are produced in the local tumor environment.[5] There is also evidence that cancer and inflammation are linked. Inflammation is initiated when innate immune cells recognize an infection or tissue wound. In addition, immune and inflammatory responses are triggered when toll-like receptors (TLRs) recognize invading pathogens. TLR recognition of lipopolysaccharide (LPS) by MD-2, CD14, and LPS binding protein (LBP) leads to the activation of cellular signaling.[6]

Nitric oxide (NO) is a messenger molecule with multiple roles in vasodilatation, neurotransmission, as well as anti-cancer and anti-inflammatory activities. Hence, compounds that modulate NO might have potential therapeutic applications in treating a number of inflammatory diseases.[7] When cells are damaged, inducible nitric oxide synthase (iNOS) and COX-2 are produced, which eventually leads to the production of NO and prostaglandins (PGs).[8] COX-2 provides a new target in the prevention of inflammatory disease and COX-2 expression is closely associated with the activities of many intracellular signaling molecules, including mitogen-activated protein kinase (MAPK).[9] Jun-N terminal kinases (JNKs) are activated by numerous environmental stresses, such as UV and gamma radiation, ceramides, inflammatory cytokines, and growth factors.[10] JNK belongs to the MAPK family, along with ERK and p38 MAPK. The phosphorylation of ERK, p38 MAPK, and JNK contributes to the phosphorylation of IκB proteins and translocation of NF-κB in the nucleus.

Among the various bioactive compounds found in food, flavonoids are considered to have multiple effects, including antioxidant, antiviral, antibacterial, anti-inflammatory, and anticancer activities.[11] Flavonoids have also been shown to inhibit the growth of tumors in various cancer cell types. For example, the flavonoid quercetin has been shown to suppress prostate cancer cell colony formation.[12] Flavonoids are known to associate with the ATP-binding sites of tyrosine and serine kinases, and eventually inhibit their activity.[13] A

Abbreviations: **1**, 3,6,3'-trihydroxyflavone; COX, cyclooxygenase; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate; iNOS, inducible nitric oxide synthase; JNK, Jun-N terminal kinase; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MIP, macrophage inflammatory protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; NO, nitric oxide; PGs, prostaglandins; RT-PCR, reverse-transcription polymerase chain reaction; SRC-1, steroid receptor coactivator-1; TLR, toll-like receptor; TNF, tumor necrosis factor

number of studies have examined the role of flavonoids in modulating inflammatory responses *in vitro*.[14] Among them, quercetin, apigenin, luteolin, naringenin, and kaempferol have been found to exhibit anti-inflammatory activities by inhibiting NO production in lipopolysaccharide (LPS)- or cytokine-stimulated macrophages.[15] We previously reported novel anti-inflammatory activities of amentoflavone, isolated from *Ginkgo biloba* and *Hypericum perforatum*, and systematically determined the signaling cascade that regulated TLR4 and p38 MAPK.[16] Protein kinases have been studied as important drug targets and several kinase inhibitors have been used to treat the human diseases including cancer, cardio-vascular disorders, and inflammation. Among protein kinases, suppression of JNKs might present medical benefits in human diseases since JNKs play a crucial role in obesity and insulin resistance targeting type II diabetes.[17] In previous study, to identify an useful small molecule kinase inhibitors, four flavonoids including quercetagetin, quercetin, myricetin, and kaempferol, were investigated activities by an *in vitro* kinase screening system. Among tested flavonoids, only quercetagenin significantly inhibited JNK1 activity. The UVB-stimulated phosphorylation of c-Jun, AKT, and GSK38 were inhibited by quercetagetin through inhibition of AP-1 and NF-κB. Moreover, tumor volume was significantly reduced in UVB-exposed mice by quercetagetin treatment. They suggested binding model of quercetagetin and JNK1 which quercetagetin serves as ligand for the ATP-binding site of JNK1. These provide that quercetagetin might be a potent inhibitor of JNK1 and PI3-K accompanying biochemical, cell-based, and animal model study.[18] We also determined that amentoflavone has novel anticancer activities toward MCF-7 and HeLa cells, and demonstrated that its effect is mediated through stimulation of human peroxisome proliferator-activated receptor γ (hPPARγ) and regulation of phosphatase and tensin homolog (PTEN).[19]

In our previous study, we discovered flavonoids with antibacterial activities against methicillin-resistant *Staphylococcus aureus*, including 3,6-dihydroxyflavone, which serves as an inhibitor of *Enterococcus faecalis* KAS III (efKAS III).[20] We proposed that 3,6-dihydroxyflavone is a potent inhibitor of efKAS III, with potent antibacterial activities against *E. faecalis*.[21] We also demonstrated that 3,6-dihydroxyflavone is an agonist of hPPARγ, which can regulate the proliferation, apoptosis, and differentiation of various human cancer cells, with cytotoxic effects on human cervical and prostate cancer cells.[22]

In this study, we investigated the structure and activity relationship of 3,6,3′-trihydroxyflavone (**1**) with respect to its anti-inflammatory activities. **1** has one more hydroxyl group at the 3′ position compared to 3,6-dihydroxyflavone. We investigated the effect of **1** on the induction of inflammation and its mechanisms of action in mouse macrophages. Studies were also performed to examine the interactions between **1** and JNK1 by using fluorescence quenching analysis and docking studies, to confirm the role of **1** in directly modulating JNK1.

Methods

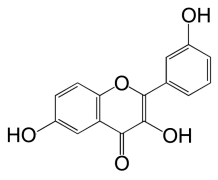
Reagents. 1 (Fig. 1) was obtained from Indofine Chemical Company (Hillsborough Township, NJ, USA) and was dissolved in H_2O:DMSO (9:1, v/v) to a concentration of 10 mg/mL (stock solution).

Cytotoxicity Against Mammalian Cells. The mouse embryonic fibroblast NIH/3T3 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The cytotoxicity of **1** in NIH/3T3 cells was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as reported previously.[23] The absorbance at 570 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell survival, expressed as a percentage, was calculated as the ratio of the absorbance at 570 nm of cells treated with **1** to the absorbance of control cells.

Quantification of Nitrite Production in LPS-stimulated RAW264.7 Cells. Nitrite accumulation in culture media was used as an indicator of NO production.[24] Raw264.7 cells were plated at a density of 1×10^5 cells/mL and stimulated with LPS (20 ng/mL) from *Escherichia coli* O111:B4 (Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of **1** for 24 h. Isolated supernatant fractions were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). The nitrite production was determined by measuring the absorbance at 540 nm and converted to nitrite concentrations by reference to a standard curve generated with $NaNO_2$.

Quantification of Inflammatory Cytokines, Mouse Tumor Necrosis Factor (mTNF)-α and Mouse Macrophage Inflammatory Protein (mMIP)-2, in LPS-stimulated RAW264.7 Cells. Antibodies against mTNF-α and mMIP-2 were used for immobilization on immune plates and utilized in enzyme-linked immunosorbent assays (ELISAs), as reported previously.[25]

Reverse Transcription-polymerase Chain Reaction (RT-PCR). RAW264.7 cells were stimulated without (negative control) or with 20 ng/mL LPS in the presence or absence of **1** for 3 h. Competitive RT-PCR was performed as described previously,[26] with minor modifications. The targets were amplified from the reverse transcribed cDNA by PCR with the following specific primers: TNF-α, 5'-GTT CTG TCC CTT TCA CTC ACT G-3' (sense) and 5-GGT AGA GAA TGG ATG AAC ACC-3' (antisense); MIP-1, 5'-ATG AAG CTC TGC GTG TCT GC-3' (sense) and 5'-TGA GGA GCA AGG ACG CTT CT-3' (antisense); MIP-2, 5'-ACA CTT CAG CCT AGC GCC AT-3' (sense) and 5-CAG GTC AGT



3,6,3'-trihydroxyflavone (1)

Figure 1. Chemical structure of the flavonoid 3,6,3′-trihydroxflavone.

TAG CCT TGC CT-3' (antisense); and IL-1β, 5'-CTG TCC TGA TGA GAG CAT CC-3' (sense) and 5'-TGT CCA TTG AGG TGG AGA GC-3' (antisense). The primers for glyceraldehyde 3-phosphate (GAPDH), used as an internal control, were 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5-TCC ACC ACC CTG TTG CTG TA-3' (antisense). PCR was performed using the following cycling conditions: 94 °C for 5 min; followed by 25 cycles of 94 °C for 1 min, 55 °C for 1.5 min and 94 °C for 1 min; and a final extension step of 72 °C for 5 min.

Western Blotting. Proteins were isolated from LPS-stimulated RAW264.7 cells in the presence or absence of **1**. Proteins were then detected as reported previously,[27] using the following antibodies: phospho-ERK (1:2000; Cell Signaling Technology, Beverly, MA, USA), phospho-p38 (1:2000, Cell Signaling Technology), JNKs (1:1000, Cell Signaling Technology), and β-actin (1:5000, Sigma-Aldrich). The relative amount of protein associated with each antibody was quantified using ImageJ (NIH, Bethesda, USA).

Construction of JNK1 Expression Plasmids. JNKs have numerous isoforms, including JNK1, JNK2, and JNK3, which have been identified in mammals. JNK1 and JNK2 are expressed ubiquitously in the cells and tissues of mammals, while JNK3 is found primarily in the brain.[28] To express JNK1, the C-terminal truncated form of human JNK1α1 (residues 1–364) was cloned into the pET21b expression vector (Novagen) and expressed in *E. coli* with a 6×His-tag at the C-terminus. JNK1 was then purified as reported previously.[29]

Fluorescence Quenching. We titrated our compound, **1**, to a 10 μM JNK1 protein solution in 50 mM sodium phosphate buffer containing 100 mM NaCl at pH 8.0, with a final JNK1:**1** ratio of 1:10. The sample was placed in a 2 mL cuvette, with excitation and emission path lengths of 10 nm. Using tryptophan emission, we determined the fluorescence quantum yields of JNK1 and **1**. The methods were performed as described previously.[30]

Docking Study. Using the X-ray crystallography structure of JNK1 (3v3v. pdb), we defined the ATP-binding site of JNK1. **1** was docked to JNK1 using CDOCKER, a CHARMm-based molecular dynamics (MD) method for ligand-docking in Discovery Studio modeling (Accelrys Inc., San Diego, CA, USA). This algorithm assumes a rigid protein and permits only the ligand to be flexible. The Input Site Sphere parameter specifies a sphere around the center of the binding site, where the CDOCKER experiment is to be performed. The center of the sphere is used in the CDOCKER algorithm for initial ligand placement. The MD-simulated annealing process is performed using a rigid protein and flexible ligand. The final minimization step is applied to the ligand's docking pose. The minimization consists of 50 steps of steepest descent followed by up to 200 steps of conjugated gradient by using an energy tolerance of 0.001 kcal/mol.[31]

Result and Discussion

Cytotoxicity in NIH/3T3 Cells. In order to examine the anti-inflammatory activities of **1** and to investigate its mechanism of action *in vitro*, we first determined the nontoxic concentration of **1**. We investigated its cytotoxicity using a MTT assay (Fig. 2). Interestingly, concentrations of up to 100 mM did not affect the survival of NIH/3T3 cells. These results clearly demonstrated that **1** has very low cytotoxicity against NIH/3T3 cells.

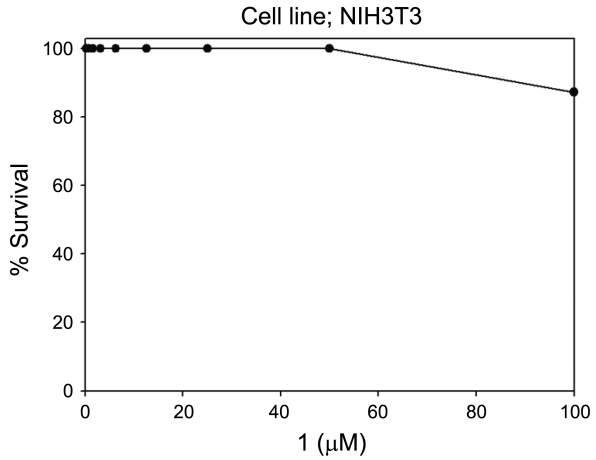


Figure 2. Cytotoxicity of 3,6,3′-trihydroxflavone (**1**) Concentration-response curves of **1** for cytotoxicity toward mouse embryonic fibroblast NIH/3T3 cell.

Effects of 3,6,3'-Trihydroxyflavone (1) on Nitrite Production in LPS-stimulated RAW264.7 Cells. To determine whether **1** inhibited LPS signaling and inflammatory cascade, we investigated nitrite production in LPS-stimulated RAW264.7. As shown in Figure 3(a), we found that **1** inhibited NO production from LPS-stimulated RAW264.7 cells at non-toxic concentrations. Treatment with 10 μM and 20 μM of **1** led to 59% and 69% inhibition of NO production, respectively, when compared to that in macrophages that were not treated with **1**.

Effects of 3,6,3'-Trihydroxyflavone (1) on Inflammatory Cytokines (mTNF-α and mMIP-2) Production in LPS-stimulated RAW264.7 Cells. We further tested LPS-induced inflammatory cytokine production in RAW264.7 cells. Mouse TNF-α and MIP-2 levels were directly measured using ELISA. Quantitative analysis revealed that 10 μM and 20 μM of **1** inhibited TNF-α production by 58% and 65%, respectively, and mMIP-2 production by 44% and 78% when compared with non-treated LPS-stimulated cells (Fig. 3(b) and (c)).

Effects of 3,6,3'-Trihydroxyflavone (1) on mRNA Expression in LPS-stimulated RAW264.7 Cells. We investigated the expression of inflammation-related cytokines, including TNF-α, MIP-1, MIP-2, and mouse interleukin (IL)-1β by using RT-PCR. When 20 ng/mL of LPS was used to treat RAW264.7 cells, the gene expression of all tested cytokines increased compared with that of non-LPS-stimulated RAW264.7 cells (Fig. 4(a)). The expression of TNF-α was inhibited by 40% in RAW264.7 cells treated with 10 mM of **1** and LPS while the expression of MIP-1 was inhibited by 21%. In addition, the expression of MIP-2 and

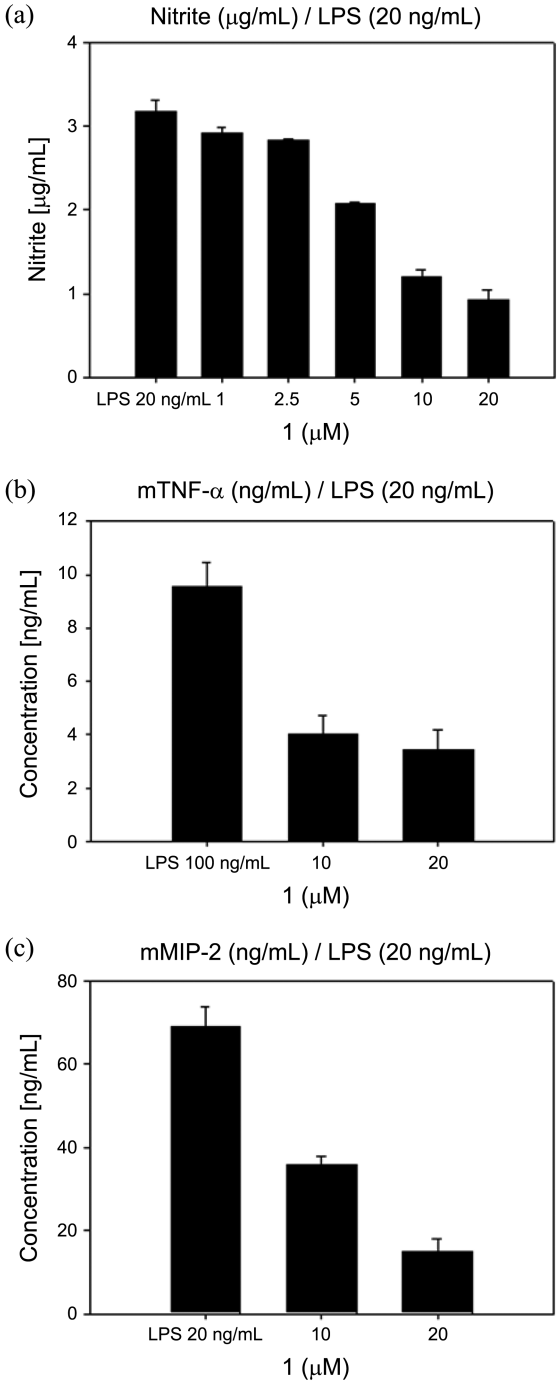


Figure 3. Effects of 3,6,3′-trihydroxflavone (**1**) on nitrite production, inflammatory cytokines in LPS-stimulated RAW264.7 cells. (a) Inhibition of nitrite production by **1** in LPS-stimulated RAW264.7 cells. NO production was measured using the Griess reagent. (b) Inhibition of mTNF-α production by **1** in LPS-stimulated RAW264.7 cells. (c) Inhibition of mMIP-2 production by **1** in LPS-stimulated RAW264.7 cells.

IL-1β mRNA was effectively decreased by 31% and 30%, respectively, compared to the mRNA levels in LPS-stimulated cells.

Effects of 3,6,3'-Trihydroxyflavone (1) on Phosphorylation of MAPKs Expression in LPS-stimulated RAW264.7 Cells. Along with RNA expression, we further tested the

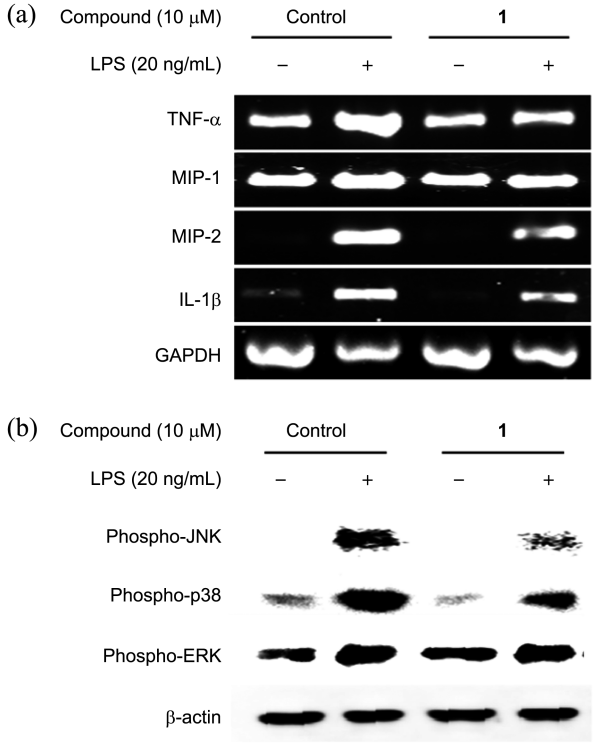


Figure 4. Effect of 3,6,3′-trihydroxflavone (**1**) on LPS induced inflammatory cytokine gene expression and MAPK phosphorylation. (a) Effects of **1** on LPS-induced expression of inflammatory cytokines in LPS-stimulated RAW264.7 cells. Total RNA was analyzed for the expression of *Tnf-α*, *Mip-1*, *Mip-2*, *Il-1β*, and *Gapdh* mRNA by RT-PCR. *Gapdh* mRNA was used as an internal control. (b) Effects of **1** on phospho-JNK, phospho-p38, and phospho-ERK in LPS-stimulated RAW264.7 cells. phospho-JNK, phospho-p38, phospho-ERK, and β-actin (loading control) were determined by western blot analysis using specific antibodies. The relative mRNA expression was quantified using image J (NIH, Bethesda, MD, USA).

phosphorylation of three MAPKs, ERK, p38 MAPK, and JNK proteins, by using western blotting (Fig. 4(b)). RAW264.7 cells were treated with **1** with or without LPS, and western blotting was performed to detect phosphorylation of ERK, p38 MAPK, and JNK. Stimulation of RAW264.7 cells with LPS significantly increased the phosphorylation of ERK, p38 MAPK, and JNK. As shown in Figure 4(b), treatment with 10 mM of **1** for 3 h repressed the phosphorylation of ERK, p38 MAPK, and JNK. **1** repressed the phosphorylation of JNK by 60% when compared to the levels observed in LPS-treated cells. In addition, **1**-treated macrophages had levels of phospho-p38 and phospho-ERK that were lower by 48% and 7%, respectively, compared with the levels observed in non-treated cells. **1** was found to inhibit NO production by decreasing ERK, p38, and JNK activation. Thus, ERK, p38, and JNK pathways might be potential targets for therapeutics against inflammatory disease.

Binding Affinities of 3,6,3′-Trihydroxyflavone (1) for JNK1. Since **1** was more potent in inhibiting JNK in our western blot analysis and JNKs are significantly related with chronic disease conditions such as inflammation, obesity,

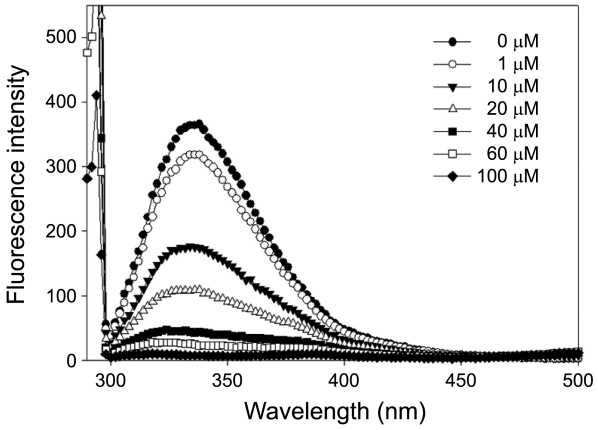


Figure 5. Fluorescence spectra of JNK1 in the presence of 3,6,3′-trihydroxflavone (**1**) at pH 7.0. The sample was excited at 290 nm, and emission spectra recorded for light scattering effect at 290 to 600 nm.

and cancer, we investigated the interactions between 1 and JNK1. We assessed the binding constants of **1** binding to JNK1 by performing fluorescence quenching experiments. As shown in Figure 5, the fluorescence intensity was altered with increases in **1** concentration. **1** exhibited a binding affinity to JNK1 of $1.568 \times 10^8\ M^{-1}$.

Docking Results of 3,6,3′-Trihydroxyflavone (1) and JNK1. To determine the potency of **1** as an inhibitor of JNK1, we performed docking studies with **1** and JNK1 in order to develop a binding model. Overall structure and docking model of JNK1 and **1** are represented in Figure 1. The ATP-binding site is well characterized in JNK structure. The structure of JNK1 contains two domains or lobes which are β-sheet rich N-terminal domain (residues 9 to 112 and 347 to 363) and α-helix rich C-terminal domain (residues 113 to 337). ATP-binding site is near these two domain interface.[32] The results from the docking studies showed that the side chains of Lys55 and Asn114 of JNK1 respectively formed a network of hydrogen bonds with the 6-hydroxy group of the A-ring and the 3-hydroxy group of the C-ring of **1** at the ATP active site, while the oxygen at the 3' position of the B-ring formed a hydrogen bond with the side chains of Met111. Also, JNK1 includes two possible hydrophobic interactions. One is formed by V40 and L168 of JNK1 and second hydrophobic site is formed by I32 and V158 of JNK1. On the basis of these hydrophobic interactions, **1** was involved in two additional possible hydrophobic interactions at the ATP binding site, including Ile32, Val40, Val158, and Leu168. The B-ring of **1** participated in a hydrophobic interaction with Ile32 and Val158, and the A-ring and C-ring of **1** could form hydrophobic interactions with Val40 and Leu168. These interactions could contribute to the increase in the binding affinity of **1** for JNK1. The results of this study will be helpful in understanding the mechanism of **1** against JNKs.

Conclusion

We found that **1** could regulate LPS-induced pro-inflammatory cytokine production by inhibiting p38-, ERK-, and JNK-dependent pathways in mouse macrophages. Also it was found that **1** was a potent inhibitor of JNK dependent inflammation in mammalian cells without cytotoxicity. Our binding studies revealed that hydrogen binding interactions as well as extensive hydrophobic interactions between **1** and JNK1 might be essential for the potency of **1** as an inhibitor of JNK1, resulting in its anti-inflammatory effect. Further studies are warranted to study its anti-cancer activities as well as its mechanism of action in detail.

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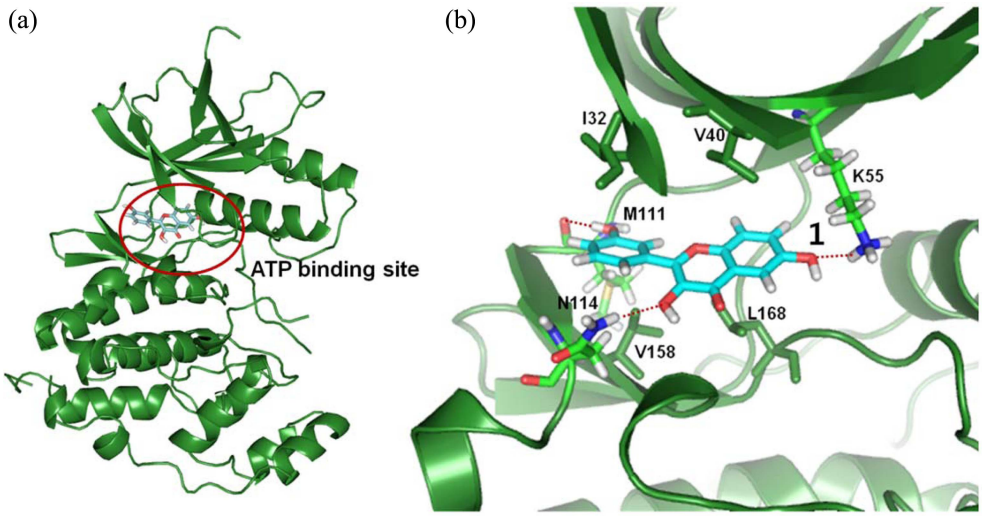

Figure 6. Docking model of 3,6,3′-trihydroxflavone (**1**) and JNK1. (a) Overall structure of human **1** and JNK1. ATP-binding site marks red circle. (b) Binding model of **1** and JNK1. Hydrogen bonds are depicted as red dashed lines.

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