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Inhibitory Activities of Kojyl Thioether Derivatives against Nitric Oxide Production Induced by Lipopolysaccharide

Ho Sik Rho,[*] Dae Sung Yoo,[†] Soo Mi Ahn, Myung Kyoo Kim,[‡] Dong Ha Cho,[†] and Jae Youl Cho[†,*]

R & D Center, AmorePacific Corporation, Yongin, 446-729, Korea. [*]E-mail: thiocarbon@freechal.com
[†]College of Biomedical Science, Kangwon National University, Chuncheon 200-701, Korea. [*]E-mail: jaecho@kangwon.ac.kr
[‡]Samkyung Costech Co., Ltd, Suwon 443-734, Korea
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Inflammation[1] is involved in the pathogenesis of numerous diseases such as chronic inflammation, cancer, rheumatoid arthritis, and autoimmune diabetes. Macrophages play a pivotal role in inflammation by producing various mediators. One such primary inflammatory mediator, nitric oxide (NO), is produced enzymatically by inducible nitric oxide synthase (iNOS).[2] The over-production of NO by iNOS is a critical event required for the development of inflammatory diseases. Accordingly, there have been substantial efforts in the pharmaceutical industry to discover potent NO inhibitors. Kojic acid, 5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one, is produced from carbohydrate sources in an aerobic process by a variety of microorganisms. It shows broad biological activities such as inhibition of tyrosinase,[3] chelation of metal ions,[4] scavenging of free radicals and prevention of photodamage.[5] However, studies on kojic acid and its derivatives as NO inhibitors are rare. Recently, we reported[6] that the thioether derivative **2** containing two molecules of kojic acid strongly inhibit NO production (Fig. 1). The structure of **2** comprises three main parts: the enolic hydroxyl group, the thioether linkage, and the aromatic ring. We further investigated the structure-activity relationship (SAR) of kojyl thioether derivatives containing the aromatic moiety on the inhibition of NO production.

The synthetic pathways are shown in Schemes 1 and 2. Kojic acid **1** was reacted with thionyl chloride to afford kojyl chloride **3**. Kojyl chloride **3** was reacted with thiols in the presence of triethylamine in THF to afford the corresponding thioether derivatives (**4a**-**4m**). The thioether derivatives (**4e**, **4f**, and **4g**) were reacted with MCPBA (*m*-chloroperbenzoic acid) in methylene chloride to produce sulfoxide derivatives (**5e**, **5f**, and **5g**). Sulfone derivatives (**6e**, **6f**, and **6g**) were obtained by treating the thioether derivatives (**4e**, **4f**, and **4g**) with oxone in a mixture of MeOH/H_2O.

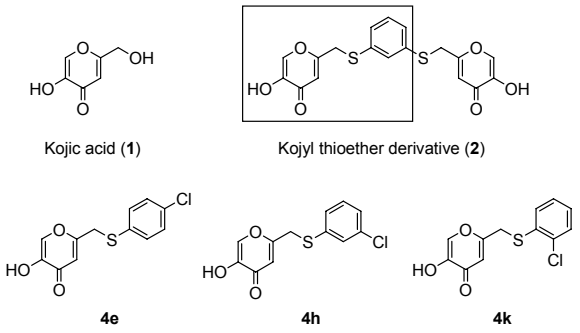
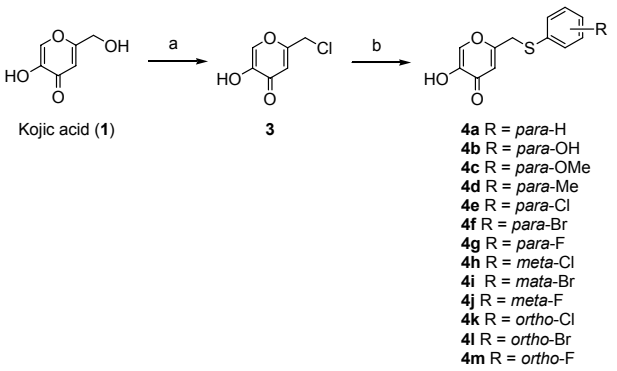


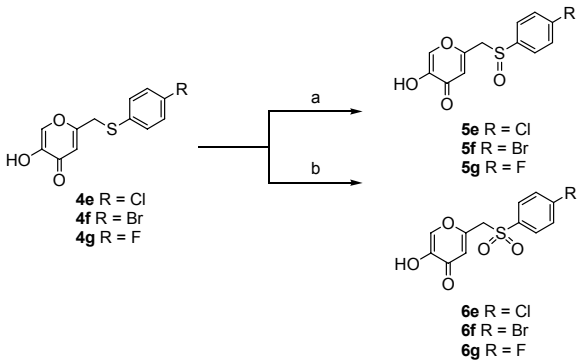
Figure 1. Structures of kojic acid and its thioether derivatives.

We investigated the cytotoxicities and inhibitory activities of kojic acid derivatives against NO production[7] induced by lipopolysaccharide (LPS) in macrophages. The results are shown in Table 1.

The IC_{50} value of kojic acid was 89.41 μM. 5-Hydroxy-2-((phenylthio)methyl)-4-*H*-pyran-4-one (**4a**) exhibited activity similar to that of kojic acid. When hydroxyl or methoxy groups were added at the *para* position of the aromatic ring, the inhibitory activities was slightly enhanced. (IC_{50} of **4b** = 70.19 μM



Scheme 1. Reaction conditions; (a) $SOCl_2$, DMF, rt; (b) benzenethiols, triethylamine, THF, rt



Scheme 2. Reaction conditions; (a) MCPBA, methylene chloride, rt; (b) oxone, MeOH/H_2O, rt

Table 1. NO inhibitory activities of kojic acid derivatives

Compounds	Inhibitory activity [IC_{50}[a] (μM)]	
	NO	Cytotoxicity
Kojic acid	89.41 ± 3.42	> 100
4a	82.48 ± 11.87	> 100
4b	70.19 ± 9.25	> 100
4c	78.01 ± 4.65	> 100
4d	> 100	> 100
4e	23.95 ± 0.27	> 100
4f	25.28 ± 0.47	> 100
4g	58.04 ± 2.62	> 100
4h	52.83 ± 2.88	> 100
4i	36.65 ± 4.21	> 100
4j	74.53 ± 2.23	> 100
4k	81.92 ± 3.88	> 100
4l	74.53 ± 1.23	> 100
4m	> 100	> 100
5e	> 100	> 100
5f	> 100	> 100
5g	> 100	> 100
6e	> 100	> 100
6f	> 100	> 100
6g	> 100	> 100

[a]Values were determined from logarithmic concentration-inhibition curves and are given as means of three experiments.

and IC_{50} of **4c** = 78.01 μM). However, the 4-methyl-substituted derivative **4d** showed decreased activity. All halogen-substituted derivatives (**4e**, **4f**, and **4g**) were effective at inhibiting NO production with the substituent effect decreasing in the orders -Cl > -Br > -F. The inhibitory activities were also influenced by the position of halogen. The activity of 4-chloro compound (**4e**) was more potent than those of 3-chloro compound (**4h**) and 2-chloro compound (**4k**). In case of bromo and fluoro derivatives, similar results were obtained. Among tested compounds, 5-hydroxy-2-((4-chlorophenylthio)methyl)-4*H*-pyran-4-one (**4e**) exhibited the most potent inhibitory activity (IC_{50} = 23.95 μM). Its level of activity is four times more potent than that of kojic acid. In the next set of experiments, we changed the sulfur functional group of the compounds (**4e**, **4f**, and **4g**). Interestingly, sulfoxides (**5e**, **5f,** and **5g**) and sulfones (**6e**, **6f**, and **6g**) exhibited decreased activity. These results indicated that the inhibitory activity of kojic acid derivatives depended on the substituent of aromatic ring (-Cl > -Br > -F > -OH > -OMe > -Me), position (*-para* > *-meta* > *-ortho*) and the sulfide linkage.

To elucidate the mechanism underlying the inhibition of NO production, we examined the effects of **4e** on the expression of iNOS mRNA in LPS-activated RAW 264.7 cells.[8] During RT-PCR analysis, the mRNA of iNOS was induced by treatment with 1 μg/mL LPS for 6 h. Treatment with **4e** suppressed the expression of iNOS mRNA significantly at concentrations of 50 and 100 μM (Fig. 2). These results suggested that the inhibition of NO production by compound **4e** was due to the suppression of iNOS mRNA.

In conclusion, we synthesized a series of kojic acid thioether derivatives (**4a-4m**), sulfoxide derivatives (**5e**, **5f**, and **5g**), and

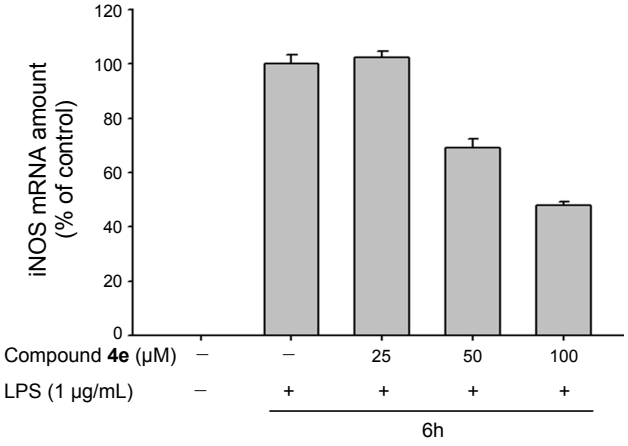


Figure 2. Effect of compound **4e** on the expression of iNOS mRNA in LPS-activated macrophages.

sulfone derivatives (**6e**, **6f**, and **6g**) and evaluated their inhibitory activities against NO production in LPS-activated macrophages. Several thioether derivatives showed promising inhibitory activities against NO production. The SAR study indicated that modification of the sulfur linkage and the halogen-substituted aromatic ring highly influenced the inhibitory activity of NO production. Among the tested compounds, 5-hydroxy-2-((4-chlorophenylthio)methyl)-4*H*-pyran-4-one (**4e**) exhibited the most potent inhibitory activity (IC_{50} = 23.95 μM). RT-PCR analysis suggested that compound **4e** inhibited NO production through the suppression of iNOS mRNA expression.

Experimental Section

Typical procedure.

5-Hydroxy-2-((4-methylphenylthio)methyl)-4*H*-pyran-4-one (4d): To a stirred solution of kojyl chloride **3** (4.8 g, 30 mmol) and trietylamine (4.0 g, 40 mmol) in THF (100 mL) under N_2 was added 4-methylbenzenethiol (4.8 g, 33 mmol). The reaction mixture was stirred for 10 h at room temperature, after which THF was evaporated in vacuo. The residue was extracted with ethyl acetate (300 mL), washed with water. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl acetate-hexane to give **4e** (6.0 g) in 75 % yields.

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.14 (s, 1H), 8.01 (s, 1H), 7.28 (d, 2H, J = 7.5 Hz), 7.15 (d, 2H, J = 7.5 Hz), 6.18 (s, 1H), 4.07 (s, 2H), 2.26 (s, 3H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 173.4, 163.7, 145.5, 139.70, 139.66, 136.7, 130.5, 129.91, 129.73, 112.52, 112.42, 34.9, 20.5. FAB MS: (m/e) 249 $[M+1]^+$.

5-Hydroxy-2-((4-chlorophenylthio)methyl)-4*H*-pyran-4-one (4e): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.13 (s, 1H), 8.01 (s, 1H), 7.25-7.43 (m, 4H), 6.27 (s, 2H), 4.16 (s, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 173.5, 163.5, 145.7, 139.8, 132.9, 131.74, 131.50, 129.1, 112.7, 34.3. FAB MS: (m/e) 269 $[M+1]^+$.

5-Hydroxy-2-((4-bromophenylthio)methyl)-4*H*-pyran-4-one (4f): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.18 (s, 1H), 8.02 (s, 1H), 7.50 (d, 2H, J = 8.4 Hz), 7.35 (d, 2H, J = 8.4 Hz), 6.27 (s, 1H), 4.18 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.4,

163.3, 145.6, 139.78, 139.74, 133.5, 131.88, 131.51, 119.9, 112.61, 112.51, 34.0. FAB MS: (m/e) 313 $[M+1]^+$.

5-Hydroxy-2-((4-fluorophenylthio)methyl)-4*H*-pyran-4-one (4g): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.16 (s, 1H), 8.01 (s, 1H), 7.42 (m, 2H), 7.21 (m, 2H), 6.18 (s, 1H), 4.10 (s, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 173.4, 163.5, 145.5, 139.7, 133.21, 133.09, 129.0, 116.29, 116.00, 112.59, 112.51, 35.3. FAB MS: (m/e) 253 $[M+1]^+$.

5-Hydroxy-2-((3-chlorophenylthio)methyl)-4*H*-pyran-4-one (4h): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.17 (s, 1H), 8.01 (s, 1H), 7.47 (s, 1H), 7.31-7.36 (m, 3H), 6.31 (s, 1H), 4.23 (s, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 173.4, 163.2, 145.6, 139.8, 136.5, 133.6, 130.6, 128.2, 127.6, 126.6, 112.6, 33.5. FAB MS: (m/e) 269 $[M+1]^+$.

5-Hydroxy-2-((3-bromophenylthio)methyl)-4*H*-pyran-4-one (4i): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.19 (s, 1H), 7.98 (s, 1H), 7.56 (s, 1H), 7.35 (m, 2H), 7.23 (m, 1H), 6.26 (s, 1H), 4.19 (s, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 173.4, 163.5, 145.5, 139.7, 133.2, 133.1, 129.0, 116.3, 116.0, 112.6, 112.5, 35.3. FAB MS: (m/e) 313 $[M+1]^+$.

5-Hydroxy-2-((3-fluorophenylthio)methyl)-4*H*-pyran-4-one (4j): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.15 (s, 1H), 8.01 (s, 1H), 7.27-7.37 (m, 2h), 7.26 (d, 1H, J = 7.8 Hz), 7.06 (m, 1H), 6.33 (s, 1H), 4.23 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.5, 163.8, 145.6, 139.8, 136.7, 130.8, 124.9, 115.60, 115.29, 113.6, 112.6, 33.5. FAB MS: (m/e) 253 $[M+1]^+$.

5-Hydroxy-2-((2-chlorophenylthio)methyl)-4*H*-pyran-4-one (4k): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.15 (s, 1H), 8.02 (s, 1H), 7.51 (m, 2H), 7.25-7.34 (m, 2H), 6.34 (s, 1H), 4.24 (s, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 173.4, 162.9, 145.6, 139.8, 133.2, 132.4, 129.64, 129.51, 127.84, 127.80, 112.6, 32.9. FAB MS: (m/e) 269 $[M+1]^+$.

5-Hydroxy-2-((2-bromophenylthio)methyl)-4*H*-pyran-4-one (4l): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.19 (s, 1H), 8.03 (s, 1H), 7.62 (d, 2H, J = 8.1 Hz), 7.46 (d, 2H, J = 8.1 Hz), 7.38 (m, 1H), 7.19 (m, 1H), 6.36 (s, 1H), 4.24 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.4, 162.9, 145.6, 139.8, 135.3, 132.8, 129.11, 128.31, 127.85, 122.7, 112.6, 33.3. FAB MS: (m/e) 313 $[M+1]^+$.

5-Hydroxy-2-((2-fluorophenylthio)methyl)-4*H*-pyran-4-one (4m): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.07 (s, 1H), 7.94 (s, 1H), 7.44 (m, 1H), 7.29 (m, 1H), 7.11-7.22 (m, 2H), 6.14 (s, 1H), 4.05 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.5, 163.2, 145.6, 139.8, 132.9, 129.9, 125.1, 120.3, 115.88, 115.59, 112.5, 34.0. FAB MS: (m/e) 313 $[M+1]^+$.

5-Hydroxy-2-((4-chlorophenylsulfinyl)methyl)-4*H*-pyran-4-one (5e): To a solution of compound **4e** (5.4 g, 20 mmol) in methylene chloride (100 mL) at 0 °C were added m-chloroperbenzoic acid (3.8 g, 22 mmol). The reaction mixture was warmed to room temperature and stirred for 5 h, after which methylene chloride was evaporated in vacuo. The residue was extracted with ethyl acetate (300 mL), washed with water. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl acetate-hexane to give **5e** (3.5 g) in 62% yields.

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.21 (s, 1H), 7.88 (s, 1H), 7.62-7.64 (m, 4H), 6.13 (s, 1H), 4.35 (d, 1H, J = 13.5 Hz), 4.17 (d, 1H, J = 13.5 Hz). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.1, 157.2, 146.0, 141.5, 140.0, 136.2, 129.3, 126.2, 115.8, 58.0. FAB MS: (m/e) 285 $[M+1]^+$.

5-Hydroxy-2-((4-bromophenylsulfinyl)methyl)-4*H*-pyran-4-one (5f): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.21 (s, 1H), 7.89 (s, 1H), 7.80 (d, 2H, J = 8.1 Hz), 7.56 (d, 2H, J = 8.1 Hz), 6.14 (s, 1H), 4.39 (d, 1H, J = 13.5 Hz), 4.16 (d, 1H, J = 13.5 Hz). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 172.9, 157.1, 145.9, 141.8, 139.9, 132.0, 126.2, 124.8, 115.6, 57.8. FAB MS: (m/e) 329 $[M+1]^+$.

5-Hydroxy-2-((4-fluorophenylsulfinyl)methyl)-4*H*-pyran-4-one (5e): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.22 (s, 1H), 7.87 (s, 1H), 7.65 (m, 2H), 7.43 (m, 2H), 6.13 (s, 1H), 4.34 (d, 1H, J = 13.5 Hz), 4.15 (d, 1H, J = 13.5 Hz). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 172.9, 157.1, 145.8, 139.8, 138.14, 138.10, 126.85, 126.73, 116.45, 116.15, 115.57, 58.0. FAB MS: (m/e) 269 $[M+1]^+$.

5-Hydroxy-2-((4-chlorophenylsulfonyl)methyl)-4*H*-pyran-4-one (6e): To a solution of compound **4e** (5.4 g, 20 mmol) in the mixture of MeOH (100 mL) and H_2O (20 mL) was added excess of Oxone (18.5 g, 60 mmol). The reaction mixture was stirred for 10 h at room temperature, after which MeOH was evaporated in vacuo. The residue was extracted with ethyl acetate (500 mL), washed with water. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl acetate-hexane to give a **5e** (3.8 g) in 60% yields.

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.25 (s, 1H), 7.98 (s, 1H), 7.84 (d, 2H, J = 8.4 Hz), 7.75 (d, 2H, J = 8.4 Hz), 6.25 (s, 1H), 4.88 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.2, 155.7, 146.2, 140.4, 139.6, 136.9, 130.1, 129.67, 116.59, 58.53. FAB MS: (m/e) 301 $[M+1]^+$.

5-Hydroxy-2-((4-bromophenylsulfonyl)methyl)-4*H*-pyran-4-one (6f): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.32 (s, 1H), 7.98 (s, 1H), 7.89 (d, 2H, J = 8.4 Hz), 7.73 (d, 2H, J = 8.4 Hz), 6.25 (s, 1H), 4.87 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.0, 155.6, 146.1, 140.3, 137.2, 132.5, 129.9, 128.6, 116.4, 58.4. FAB MS: (m/e) 345 $[M+1]^+$.

5-Hydroxy-2-((4-fluorophenylsulfonyl)methyl)-4*H*-pyran-4-one (6g): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.23 (s, 1H), 7.97 (s, 1H), 7.89 (m, 2H), 7.53 (m, 2H), 6.24 (s, 1H), 4.86 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.1, 155.8, 146.1, 140.3, 134.35, 134.31, 131.39, 131.26, 116.83, 116.53, 116.44, 58.6. FAB MS: (m/e) 285 $[M+1]^+$.

Measurements of NO production. RAW264.7 cells (1×10^6 cells/mL) were preincubated with kojyl acid derivatives for 30 min and continuously activated with LPS (1 μg/mL) for 24 h. Nitrite in culture supernatants was measured by adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μL samples of the medium for 10 min at room temperature. OD at 570 nm (OD_{570}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). A standard curve of NO was made with sodium nitrite.

Measurements of cytotoxicity. After the preincubation of RAW264.7 cells (1×10^6 cells/mL) for 18 h, kojyl thioether derivatives (0 - 100 μM) were added to the cells and incubated for 24 h. The cytotoxic effect of kojyl thioether derivatives was

then evaluated by a conventional MTT assay. At 3 h prior to culture termination, 10 μL of the MTT solution (5 mg/mL in a phosphate buffered-saline, pH 7.4) were added and the cells were continuously cultured until termination. The incubation was halted by the addition of 15% sodium dodecyl sulfate into each well, solubilizing formazan. The absorbance at 570 nm ($OD_{570-630}$) was measured by a Spectramax 250 microplate reader.

mRNA detection by quantitative and semi-quantitative real-time reverse transcription-PCR. Total RNA from LPS-treated-RAW264.7 cells (5×10^6 cells/mL) was prepared by adding TRIzol Reagent (Gibco BRL) according to the manufacturer's protocol. The total RNA solution was stored at −70 °C until used. Semi-quantitative RT reactions were conducted using MuLV reverse transcriptase. Total RNA (1 μg) was incubated with oligo-dT15 for 5 min at −70 °C and mixed with a 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 for 60 min after the addition of MuLV reverse transcriptase (2 U). Reactions were terminated after 10 min at 70 °C, and total RNA was depleted by adding RNase H. The PCR reaction was conducted with the incubation mixture (2 μL cDNA, 4 μM 5' and 3' primers, a 10 × buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100], 250 μM of dNTP, 25 mM of MgCl2, and 1 unit of Taq polymerase [Promega, USA]). The following incubation conditions were used: a 30 s denaturation time at 94 °C, an annealing time of 30 s between 55 and 60 °C, an extension time of 45 s at 72 °C, and a final extension of 5 min at 72 °C. For real-time PCR analysis, 1 μg of RNA was submitted to reverse transcription with the Molony murine leukemia virus reverse transcriptase (Invitrogen). Two microliters of cDNA obtained for each sample were submitted to a qPCR using the SYBR green Master mix method (Applied Biosystems, Foster City, CA) in the ABO sequence detection system. The results were normalized with the 18S transcript. The primers (Bioneer, Daejeon, Korea) used in this experiment are indicated as follows: iNOS F-5'-GGAGCCTTTAGACCTCAACAGA-3' and R-5'-TGAACGAGGAGGGTGGTG-3'; GAPDH F-5'-CAATGAATACGGCTACAGCAAC-3' and 5'-AGGGAGATGCTCAGTGTTGG-3'.

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