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ARTICLE

Anti-Inflammatory and Antiproliferative Activities of Trifolirhizin, a Flavonoid from *Sophora flavescens* Roots

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Trifolirhizin, a pterocarpan flavonoid, was isolated from the roots of *Sophora flavescens*, and its chemical structure was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Its anti-inflammatory activity was examined in lipopolysaccharide (LPS)-stimulated mouse J774A.1 macrophages. Trifolirhizin not only dose-dependently inhibited LPS-induced expression of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) but also inhibited lipopolysaccharide (LPS)-induced expression of cyclooxygenase-2 (COX-2). In addition, trifolirhizin showed in vitro inhibitory effects on the growth of human A2780 ovarian and H23 lung cancer cells. These results suggest that trifolirhizin possesses potential anti-inflammatory and anticancer activities.

KEYWORDS: Anti-inflammation; IL-6; COX-2; *Sophora flavescens*; TNF-α; trifolirhizin

INTRODUCTION

Inflammation plays an important role in a wide variety of chronic human diseases such as cardiovascular diseases and cancer. It has been demonstrated that pro-inflammatory cytokines, cyclooxygenase-2 (COX-2), and free radical species interact in a complex manner in an inflammation environment (*1*). For example, tumor necrosis factor-α (TNF-α) has been shown to be one of the major cytokines that mediates many crucial events for the initiation of both acute and chronic inflammation through regulating the production of some other cytokines, up-regulation of adhesion molecule expression, and the activation of leukocyte-specific chemotactic cytokines (*2*). Interleukin-6 (IL-6) is another pro-inflammatory cytokine that promotes inflammatory events through the activation and proliferation of lymphocytes, differentiation of B cells, leukocyte recruitment, and the induction of the acute-phase protein response in the liver (*3*). Pro-inflammatory cytokines such as TNF-α and IL-6 are also interlinked with the production of some small inflammatory mediators such as NO and prostaglandin (PGE_2) and thus contribute to the inflammatory response. In addition, COX-2 has been identified as an important link in the inflammation cascade. Unlike COX-1, COX-2 is selectively induced by pro-inflammatory factors at the site of inflammation and is responsible for the generation of inflammatory prostaglandins, which result in an inflammation response and the production of pain (*4*). Inhibition of the expression and production of these powerful mediators by anti-inflammatory components might represent a possible preventive or therapeutic target and may be used to develop anti-inflammatory nutraceuticals for health promotion and disease prevention.

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The roots of *S. flavescens* (Leguminosae) have been traditionally used in East Asian countries as herbal medicine and functional food ingredient for thousands of years because of its potential health beneficial properties such as improving mental heath and anti-inflammatory, antiasthmatic, antihelminthic, free radical scavenging, and antimicrobial activities (*5*−*9*). Previous studies have isolated quinolizidine alkaloids, flavonoids, and triterpenoids from the roots of *S. flavescens* (*5*, *8*, *10*). In 2000, flavonoids isolated from *S. flavescens* showed antiproliferative activities against human myeloid leukemia HL-60 and human hepatocarcinoma HepG2 cells and induced apoptosis in both cell lines (*10*). Later in 2002, a prenylated flavonoid from this herb was able to down-regulate COX-2 in LPS-treated RAW 264.7 cells and exhibited an in vivo anti-inflammatory effect (*7*). As part of our continuous effort to develop novel nutraceuticals for functional food utilization, this study was conducted to explore the possibility of discovering additional natural anti-inflammatory flavonoids from the roots of *S. flavescens*. The anti-inflammatory activities were examined and estimated as the inhibitory capacity on LPS induced expression of the proinflammaroty cytokines TNF-α and IL-6, and COX-2 in macrophages. The pure flavonoid compound was also evaluated for its antiproliferative activity against A2780 ovarian and H23 lung cancer cells as well as its scavenging capacity against the stable 2,2-diphenyl-1-picrylhydrazyl DPPH radical.

MATERIALS AND METHODS

General Procedures and Reagents. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker-AMX 500 instrument using DMSO-d_6 as a solvent. Electrospray ionization (ESI) mass spectra were acquired in the positive ion mode on a LCQ DECA XP instrument (Thermo Finnigan, San Jose, CA, USA) equipped with an ion trap mass analyzer. Column chromatography was carried out on silica gel (200−300 mesh, Fisherscitific, US). The Mouse J774A.1 macrophage cell line was obtained from ATCC. RNAqueous total RNA isolation kit was purchased from Ambion (Austin, TX).

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High-capacity cDNA archive kit and gene expression kit were obtained from Applied Biosystems (Foster City, CA). Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories (Hercules, CA). Western Lightning Chemiluminescence Reagent Plus was from Perkin-Elmer Life Sciences (Boston, MA). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Plant Material. Roots of *Sophora flavescens* was collected from Shanxi Province, China, in October 2006, and authenticated by Dr. Zhihong Cheng.

Isolation and Separation of Trifolirhizin. Air-dried roots of *S. flavescens* were ground, and refluxed and extracted three times for 4 h with methanol using a dried material/solvent ratio of 1:10 (w/v). The supernatant was collected by filtration, and the solvent was evaporated under reduced pressure to yield a brown solid residue. The residue was subjected to a silica gel column chromatography (CC) eluted with a mixture of chloroform/methanol of increasing polarity to afford three fractions. Fraction III, eluted by a mixture of chloroform/methanol (5:1, v/v), was further separated over silica gel CC eluted with chloroform/methanol (10:1, v/v), followed by recrystallization in methanol to obtain the pure flavonoid compound, which was identified as trifolirhizin.

RNA Isolation and Real-Time Quantitative PCR. Mouse J774A.1 macrophages were pretreated with trifolirhizin (10 or 25 μM) for 2 h, then treated with lipopolysaccharide (LPS) at a final concentration of 0.5 μg/mL for 24 h. Total cellular RNA was isolated using the Ambion RNAqueous kit. Five micrograms of total RNA was used for first-strand cDNA synthesis using a High-Capacity cDNA Archive Kit. The mRNA levels of TNF-α and IL-6 were quantified using the specific gene expression assay kits for mouse TNF-α and IL-6 on iQ5 Multicolor Real-Time PCR Detection System. The mRNA values for each gene were normalized to the internal control, β-actin mRNA. The ratio of normalized mean value for each treatment group to vehicle control group (DMSO) was calculated (*11*).

Western Blot Analysis. Mouse J774A.1 macrophage cells were pretreated with trifolirhizin (100 or 200 μM) for 2 h, then treated with LPS (0.5 μg/mL) for 24 h. Total cell lysates were prepared as previously described (*12*). The protein concentration was determined using the Bio-Rad Protein Assay reagent. The total cellular proteins (10 μg) were resolved on 10% Bis-Tris gels and transferred to nitrocellulose membranes. Immunoblots were blocked overnight at 4 °C with 5% nonfat milk in Tris-buffered saline (TBS) and then incubated with antibodies to COX-2 or β-actin. Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibody and the Western Lightning Chemiluminescence Reagent Plus. The density of the immunoblot bands was analyzed using Image J computer software (NIH).

Antiproliferative Activity Estimation. The A2780 ovarian cancer or H23 lung cancer cells were plated in 96-well plates with a density of 1×10^4/well. The medium was replaced after 24 h. Following this incubation, trifolirhizin, dissolved in DMSO, was added to the wells in an appropriate series of concentrations. Ten microliters of MTT solution was added to each well. After 24 h of incubation at 37 °C in a humidified 5% CO_2 atmosphere, the absorbance at 570 nm was recorded using an ELISA plate reader. The control refers to incubations in the presence of vehicle only (DMSO, 0.5%) and was considered as 100% viable cells.

DPPH$^{\bullet}$ Scavenging Capacity. The DPPH$^{\bullet}$ scavenging capacity of trifolirhizin was evaluated using the high throughput assay described previously (*13*). Briefly, the assay was performed using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland) and 96-well plates. The reaction mixture contained 100 μL of 0.2 mM DPPH$^{\bullet}$ in ethanol and 100 μL of standards, control, blank, or trifolirhizin. The absorbance of each reaction mixture at 515 nm was measured every minute for 40 min. The level of DPPH$^{\bullet}$ scavenged was calculated as $[(A_0 - A_1/A_0)] \times 100$ (where A_0 was the absorbance of the reagent blank, and A_1 was the absorbance with trifolirhizin). All the measurements were conducted in triplicate.

Statistical Analysis. All values are expressed as the mean ± SD of three independent determinations. Student's *t*-test was employed to analyze the differences between sets of data. Statistical analyses were performed using GraphPad Pro (GraphPad, San Diego, CA). Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Isolation and Identification of Trifolirhizin. The $CHCl_3$/methanol (5:1, v/v) fraction of the methanol extract of *S. flavescens* roots was further separated by silica gel column chromatography to obtain a pure flavonoid compound. After recrystallization in methanol, needle crystals with light yellowish color were collected. The molecular formula of the compound was established as $C_{22}H_{22}O_{16}$ by ESI MS (*m*/*z* 447 $[M + H]^+$ and 469 $[M + Na]^+$). The structure of the pure compound was identified as trifolirhizin based on spectroscopic analysis including ^{1}H- and ^{13}C-NMR spectroscopy, and electrospray ionization mass spectrometry (ESI MS). The chemical structure of trifolirhizin is shown in **Figure 1**. Its ^{1}H and ^{13}C NMR data, listed in **Table 1**, agreed well with the data reported previously (*5*, *14*).

Trifolirhizin is a pterocarpan, which belongs to a special group of isoflavonoids possessing two contiguous benzofuran and benzopyran rings. It was first isolated from *Trifolium pretense* L in 1960 (*15*) and identified in *S. flavescens* by Yagi and co-workers in 1989 (*5*). Trifolirhizin has not been evaluated for its potential anti-inflammatory activity, antiproliferative property against A2780 ovarian and H23 lung cancer cells, or free radical scavenging capacity, although it has been found to possess antifungal and anti tumor properties (*5*, *14*).

It is well known that deregulated inflammation leads to the massive production of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 by macrophages. Reduced overproduction of these inflammatory cytokines might be a potential target for the development of preventive and therapeutic approaches against inflammation-related health problems. In this study, trifolirhizin was for the first time examined for its potential inhibitory effects on LPS-stimulated expression of pro-inflammatory cytokines (IL-6 and TNF-α) and COX-2 in macrophages, along with its antiproliferative activity and the potential to directly react with free radicals.

Effects of Trifolirhizin on TNF-α and IL-6 mRNA Expression in LPS-Stimulated Mouse J774A.1 Macrophage Cells. In the present study, the LPS-stimulated mRNA expression of representative pro-inflammatory molecules including TNF-α and IL-6 in mouse J774A.1 macrophage cells were examined by real-time RT-PCR. As shown in **Figure 2A** and **B**, trifolirhizin significantly inhibited the LPS-induced increase in mRNA expression of TNF-α and IL-6 in a dose-dependent manner. At a concentration of 25 μM, trifolirhizin completely inhibited the LPS-induced increase of TNF-α mRNA levels.

The effects of trifolirhizin on the production of TNF-α and Il-6 were also examined by the ELSIA method. LPS-induced cells treated with trifolirhizin showed a significant decrease in TNF-α production in a dose-dependent matter (**Figure 2C**). This was in agreement with the observation that trifolirhizin dose-dependently suppressed LPS-induced mRNA expression of TNF-α. However, no significant inhibition was observed for IL-6 production under the same experimental conditions (data not shown).

In 2003, other herbal constituents including apigenin and ginsenoside were found to inhibit the same proinflammatory metabolites (*16*). It was observed that apigenin at a high dose of 37 μM significantly inhibited only LPS-induced levels of IL-6, without any significant effect on TNF-α concentrations (*16*). It was also reported that ginsenoside Rb_1 at a dose of 84 μM completely inhibited the induction of both TNF-α and IL-6 (*16*). These treatment concentrations were much higher than the trifolirhizin dose (25 μM) that effected the same observation in TNF-α mRNA levels in the present study. It does appear that trifolirhizin may be a better inhibitor of TNF-α than ginsenoside and apigenin. However, it needs to be pointed out that a strict

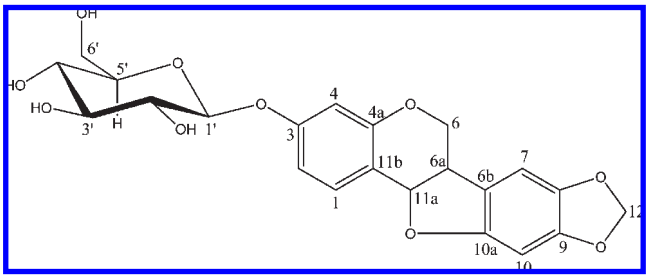

Figure 1. Chemical structure of trifolirhizin.

Table 1. NMR Spectra Data of Trifolirhizin (400 MHz, DMSO-d_6)

no. C	^{13}C NMR	^{1}H NMR
1	131.87	7.36 (d, J = 8.8 Hz)
2	110.36	6.71 (dd, J = 2.4, 8.8 Hz)
3	158.45	
4	104.00	6.55 (d, J = 2.4 Hz)
4a	156.17	
6	65.83	4.28 (dd, J = 4.0, 10.4 Hz); 3.68 (m)
6a	40.13	3.45 (m)
6b	118.23	
7	105.35	6.98 (s)
8	141.12	
9	147.47	
10	93.26	6.52 (s)
10a	153.63	
11a	77.63[a]	5.57 (d, J = 7.2 Hz)
11b	114.14	
12	101.03	5.94 (d, J = 0.8, 15.6 Hz)
1'	100.26	4.84 (d, J = 7.6 Hz)
2'	73.15	overlap
3'	76.50[a]	overlap
4'	69.63	overlap
5'	77.04[a]	overlap
6'	60.63	overlap

[a] Data can be exchanged in the column. Values in parentheses are multiplicity and coupling constants (Hz).

quantitative comparison may lead to inaccurate conclusions because of the different cells and assays used in the different studies.

Xagorari and others (*17*) also observed that luteolin and quercetin, two natural flavonoids, possessed strong inhibitory activity against these proinflammatory cytokines. The mechanism by which trifolirhizin inhibits TNF-α expression at both mRNA and protein levels and IL-6 at the mRNA level is unclear at this point, but may be related to its ability to interfere with the transcription factor NF-κB (nuclear factor kappa B). NF-κB is responsible for the expression of these proinflammatory cytokines, and a down-regulation of its activity is a plausible explanation for the observed reduction in IL-6 and TNF-α levels. Further research is required to elucidate the exact mechanism involved in the inhibitory activity of trifolirhizin.

Effects of Trifolirhizin on COX-2 Expression in LPS-Stimulated Mouse J774A.1 Macrophages. Trifolirhizin was examined for its effect on the expression of COX-2 protein in mouse J774A.1 macrophage cells stimulated with LPS. Trifolirhizin dose-dependently inhibited the LPS-stimulated COX-2 protein expression (**Figure 3**). At 0.1 and 0.2 mM concentrations, trifolirhizin suppressed LPS-induced COX-2 protein production by 14 and 28%, respectively, based on the density ratio of COX-2 versus internal control β-actin (**Figure 3**).

Expression of COX-2 may be induced by proinflammatory cytokines, stress, and growth factors. COX-2 is one of the three cyclooxygenase isozymes responsible for the production of

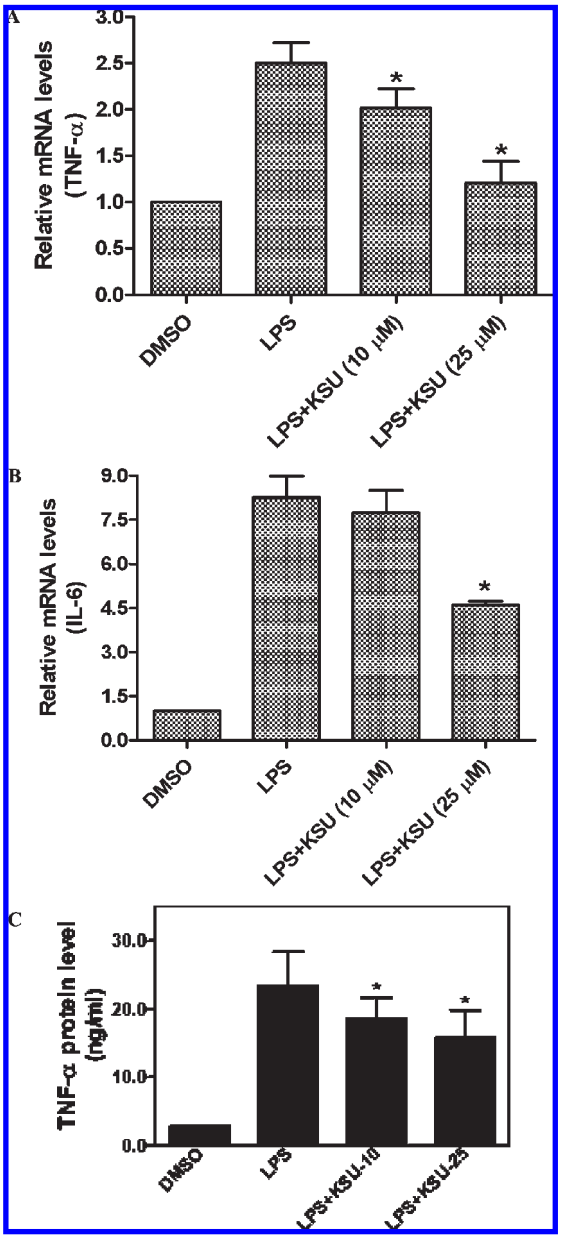

Figure 2. Effects of trifolirhizin on TNF-α (**A**) and IL-6 (**B**) mRNA levels in mouse J774A.1 macrophage cells. KSU stands for trifolirhizin. Cells (1×10^6/mL) were incubated with vehicle, LPS (0.5 μg/mL), or LPS plus indicated concentrations of trifolirhizin. The mRNA levels of TNF-α and IL-6 in the culture medium were determined as described in Materials and Methods. Each column represents the mean ± SD of three independent experiments. *P < 0.05 indicates a significant difference from the LPS treated control group.

prostaglandins. Prostaglandins are the precursors of series-2 prostanoids, which contribute significantly to the inflammatory response. Selective inhibition of COX-2 is a preferred way for controlling inflammation because of the increased risk of peptic ulcers, heart attacks, and thrombosis associated with the inhibition of the other two COX isozymes, COX-1 and COX-3. In addition to its well-established role in inflammation, COX-2 has also been implicated in human carcinogenesis. Inhibition of COX-2 may have the dual effect of controlling both inflammation and cancer and is the mode of operation of coxibs, a class of NSAIDs. The transcription factor NF-κB is responsible for regulating the expression of COX-2. In the present study, the reduction of the LPS-induced COX-2 expression in macrophages

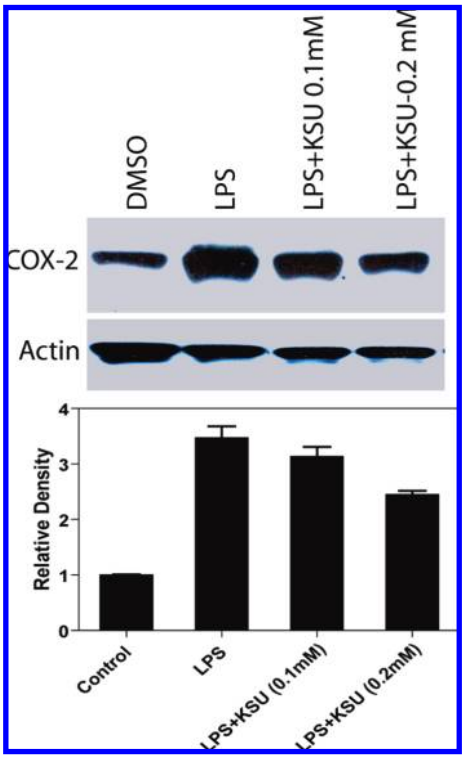

Figure 3. Effect of trifolirhizin on COX-2 expression in mouse J774A.1 macrophage cells. KSU stands for trifolirhizin. Cells (1×10^6/mL) were incubated with vehicle, LPS (0.5 μg/mL), or LPS plus indicated concentrations of trifolirhizin. COX-2 expression was determined by Western blot analysis as described in Materials and Methods.

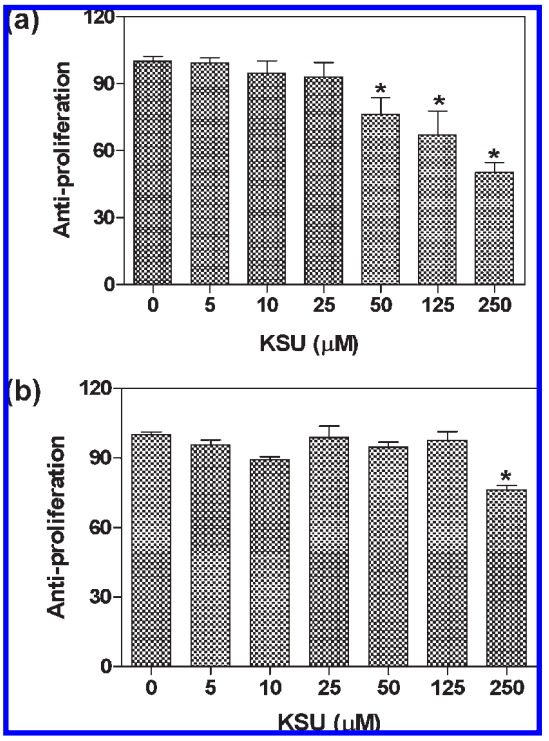

Figure 4. Dose effects of trifolirhizin on human cancer cell growth: (**A**) A237 ovarian cancer cells and (**B**) H27 lung cancer cells. Cells were exposed to serial dilutions of trifolirhizin (KSU) for 24 h. Each column represents the mean ± SD of three independent experiments.

by trifolirhizin may also be mediated through NF-κB, similar to that for the other proinflammatory cytokines studied.

Antiproliferative Activities of Trifolirhizin. A growing body of evidence suggests a direct link between inflammation and cancer (*1*, *18*). Various steps in tumorigenesis such as cellular transformation, promotion, proliferation, and metastasis have been found to be influenced by chronic inflammation (*18*). TNF-α has been associated with the induction of reactive oxygen in the form of NO, which directly oxidizes DNA and leads to cancer development (*19*). It is well accepted that anti-inflammatory agents suppressing NF-κB or NF-κB-regulated products including TNF-α, IL-6, and COX-2 may also have potential in the prevention and treatment of cancer (*1*, *18*).

In the present study, trifolirhizin dose-dependently suppressed the expression of LPS-stimulated TNF-α, IL-6, and COX-2 in mouse macrophages (**Figures 2** and **3**), which are widely accepted in vitro models for investigating anti-inflammatory agents. It is interesting to further examine its possible antiproliferative activities since trifolirhizin suppressed human myeloid leukemia HL-60 and hepatocarcinoma HepG2 cells in a previous study (*10*). Trifolirhizin was tested against human A2780 ovarian and H23 lung cancer cells using the MTT assay since these two cell lines are commonly used in our laboratory for screening and development of novel antiproliferative agents. Trifolirhizin dose-dependently suppressed the proliferation of both A2780 ovarian and H23 lung cancer cells (**Figure 4**). When exposed to trifolirhizin with concentrations less than 50 μM, no antiproliferative activity was observed in either of the two cell lines. For A2780 ovarian cancer cells, significant antiproliferation (50% growth inhibition) was achieved with concentrations up to 100 μM. However, a significant antiproliferative effect was observed only with a trifolirhizin concentration of 250 μM for H23 lung cancer cells. These data demonstrated the potential antiproliferative activity against cancer cells and its possible selectivity.

Taking into account both antiproliferative and anti-inflammatory data from this study, trifolirhizin might decrease tumorigenesis through inhibition of the inflammatory mediators. This is consistent with results from other studies that tested the anticancer activity of natural anti-inflammatory flavonoids (*20*−*23*). Many different mechanisms have been proposed to explain these actions, but they are not mutually exclusive of each other. For example, although the results from this study seem to suggest that the antiproliferative activity of trifolirhizin may be related to its ability to inhibit the expression of some proinflammatory cytokines, another study by Aratanechemuge and others (*14*) found that the antitumor effect of trifolirhizin might be explained by its ability to induce apoptosis in human promyelotic leukemia HL-60 cells. The actual mechanism may very well be a combination of several pathways. Considering in this study that both TNF-α and IL-6 mRNA expressions were suppressed, it is consistent to suggest that trifolirhizin acts at the transcription level.

DPPH$^\bullet$ Scavenging Capacity of Trifolirhizin. The capacity of trifolirhizin to directly react with and quench free radicals was evaluated and compared with that of the crude methanol extract of *S. flavescens*. The crude extract showed DPPH$^\bullet$ scavenging capacity in a dose-dependent manner (**Figure 5a**), but no significant DPPH$^\bullet$ scavenging capacity was observed for pure trifolirhizin at concentrations up to 12 mM (**Figure 5b**).

Growing evidence suggests that free radicals and free radical mediated oxidative stress are closely correlated with the development of inflammation by increasing activation of transcription factors important in the regulation of pro-inflammatory cytokines (*24*, *25*). As a result, expression of these genes stimulates the secretion of pro-inflammatory cytokines. In the present study, no significant free radical scavenging activity was observed for trifolirhizin with concentrations of up to 12 mM. This observation

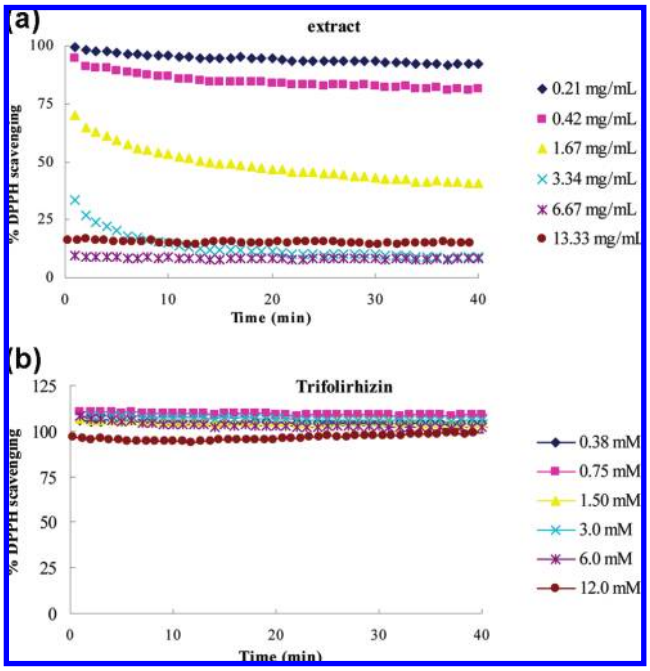

Figure 5. Dose and time effects of antioxidants-DPPH• reactions. The final concentration of DPPH• was 100 μM. (**a**) Reactions with methanol extract of *Sophora flavescens*; 0.25, 0.20, 0.17, 0.14, 0.11, and 0 represent the concentrations (mg/mL) of the extract in the initial reaction mixture. (**b**) Reactions with trifolirhizin; 1.00, 0.67, 0.50, 0.40, 0.33, 0.25, and 0 represent the concentrations of trifolirhizin in the initial reaction in mM.

was consistent with the previous studies showing that trifolirhizin might not act as an active scavenger of free radicals (*6*, *8*, *9*). This may be partially explained by the lack of phenolic hydroxyl groups in the trifolirhizin molecule (**Figure 1**). This result was also supported by another study showing that trifolirhizin did not have detectable $ONOO^-$ or DPPH• scavenging activities (*9*). Taking these previous observations together with our present data, it may be concluded that the anti-inflammatory and antiproliferative effects of trifolirhizin were not mediated through its direct interaction with free radicals.

In summary, trifolirhizin from *S. flavescens* roots was not only able to inhibit LPS-induced TNF-α, IL-6, and COX-2 expression in macrophages but also able to inhibit cancer cell growth. These findings clearly indicate that trifolirhizin possesses anti-inflammatory and anticancer activities and may have application in the prevention and treatment of inflammation and cancer.

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