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Original article:

ANTI-INFLAMMATORY EFFECT OF SELAGIN-7-O-(6''-O-ACETYL-)-B-D-GLYCOSIDE ISOLATED FROM *CANCRINIA DISCOIDEA* ON LIPOPOLYSACCHARIDE-INDUCED MOUSE MACROPHAGE RAW 264.7 CELLS

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ABSTRACT

Selagin-7-O-(6''-O-acetyl-)-β-D-glycoside, a new flavone glycoside isolated from *Cancrinia discoidea,* is known to exhibit anti-inflammatory activity in vivo. This study aimed to investigate the protection of this flavone glycoside on inflammation in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The effects of selagin-7-O-(6''-O-acetyl-)-β-D-glycoside on inflammatory cytokines and signaling pathways were analyzed by enzyme-linked immunosorbent assay, reverse transcription-polymerase chain reaction, and western blot. Results show that selagin-7-O-(6''-O-acetyl-)-β-D-glycoside protected LPS-induced macrophage RAW 264.7 cells from injury. The flavone glycoside markedly inhibited the LPS-induced production of tumor necrosis factor-α, interleukin-1β, and interleukin-6 and increased interleukin-10 release in a concentration-dependent manner. Furthermore, treatment with the flavone glycoside decreased nitric oxide and prostaglandin E2 in LPS-challenged RAW 264.7 cells. These decreases were associated with the down-regulation of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), and nuclear factor kappa B (NF-κB) activity. These findings suggest that the anti-inflammatory effects of selagin-7-O-(6''-O-acetyl-)-β-D-glycoside were associated with the adjustment of inflammatory cytokines, and attributed to the down-regulation of NF-κB and consequent suppression of the expression of iNOS and COX-2.

Keywords: Selagin-7-O-(6''-O-acetyl-)-β-D-glycoside, LPS, macrophage, cytokine, Nuclear factor-κB

INTRODUCTION

Inflammation is an initial host immune reaction mediated by inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), and related inflammatory mediators, such as nitric oxide (NO) and prostaglandin E2 (PGE2), which are produced by inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2), respectively (Becker et al., 2005; Opal and DePalo, 2000; Ronis et al., 2008). These inflammatory cytokines and mediators are essential for host survival following infection, and required for tissue repair (Birkedal-Hansen, 1993). During the development of inflammation, the concerted actions of molecular signaling determine whether inflammatory cells undergo migration, activation, proliferation, differentiation, repair, or clearance (Arita et al., 2005). An aberration of these mechanisms may favor the development of various illnesses. In-

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creased levels of free radicals have been found in many pathological conditions besides inflammation, such as cancer, ischemic disorders, and dementia (Harput et al., 2012). Thus, properly regulated inflammatory responses are necessary for healthy immune function. The use of natural products that possess anti-inflammatory properties can be of great significance in therapeutic anti-inflammatory treatments.

Flavonoids are important constituents of plants, fruits, and vegetables; they are known to possess various biological activities, including antibacterial, antifungal, antiviral, anticancer, and anti-inflammatory effects (Nijveldt et al., 2001; Amić et al., 2007; Matsuda et al., 2003). The anti-inflammatory properties of many flavonoid molecules have been studied in detail to establish and characterize their potential use as therapeutic agents in the treatment of inflammatory diseases. However, the mechanisms by which flavonoids exhibit their anti-inflammatory activity have not been clarified.

Cancrinia discoidea (Ledeb.) Poljak. (Compositae) is a historical herb used as an edible and medicinal plant for several ailments, such as inflammation, dermal ulcer, and bleeding in the Three Parallel River areas of Yunnan Province. In a previous study, we isolated numerous flavonoids from *C. discoidea*, including a new flavone glycoside, selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside (Zhu and Tian, 2010), and demonstrated that this flavone glycoside significantly ameliorates the inflammatory response in rodent models (Su et al., 2011). However, the detailed mechanism underlying the anti-inflammatory activities of the flavone glycoside is unclear. In the present work, the putative anti-inflammatory mechanism of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages was elucidated through inflammatory mediators and the nuclear factor kappa B (NF-κB) pathway.

MATERIALS AND METHODS

Materials and chemicals

Selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside (Figure 1) used for this study was isolated from *C. discoidea* (Zhu et al., 2010). The isolated compound was analyzed by liquid chromatography coupled with mass spectrometry, and its purity was found to be > 99 %. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT), and PBS were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kits for PGE2, TNF-α, IL-1β, and IL-6 were purchased from R&D Systems, Inc. (St. Louis, MO, USA), and ELISA kits for interleukin-10 (IL-10) was purchased from Biolegend (CA, USA). The antibody to NF-κB p65 was purchased from Biovision (Mountain view, CA, USA). The antibodies to COX-2 and iNOS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the antibody to β-actin was purchased from Chemicon (Temecula, CA, USA). The other chemicals and reagents used were of analytical grade.

Cell culture

RAW 264.7 cells, a murine macrophage cell line, were purchased from China Center for Type Culture Collection (Shanghai, China). Cells (2×10^5) were cultured in a 96-well plate containing DMEM (Sigma, St. Louis, MO, USA) supplemented with 10 % FBS in a CO_2 incubator (5 % CO_2) at 37 °C.

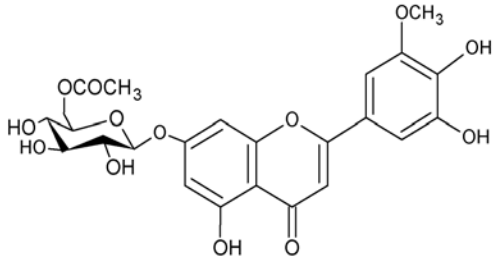

Figure 1: Structure of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside

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Cell viability

After pre-incubation for 24 h in a CO_2 incubator, the RAW 264.7 cells were pretreated with several concentrations (0, 20, 40, 80, 160, and 240 μg/mL) of the flavone glycoside for 1 h and then co-stimulated with 100 ng/mL LPS for 24 h at 37 °C. The cells were then washed twice with PBS, and incubated with 100 mL of 0.5 mg/mL MTT for 2 h to measure cell viability. The medium was discarded, and 100 mL of DMSO was added. After 30 min of incubation, absorbance at 570 nm was read using a microplate reader.

Measurement of levels of NO, PGE2, TNF-α, IL-1β, IL-6, and IL-10

RAW 264.7 cells were plated in a 12-well plate at a density of 2×10^5 cells/well and incubated for 24 h. Cultured cells were treated with various concentrations (0, 20, 40, 80, and 160 μg/mL) of the flavone glycoside for 1 h, and stimulated with 100 ng/mL LPS for 24 h. Cultured media were collected after centrifugation at 2000 g for 10 min, and stored at –80 °C until analysis. The nitrite concentration in the cultured media was measured as an indicator of NO production, according to the Griess reaction (Kim et al., 1995). Levels of PGE2, IL-1β, IL-6, IL-10, and TNF-α in cultured media were quantitated by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells placed in a 12-well plate were pretreated with various concentrations (0, 20, 40, 80, and 160 μg/mL) of the flavone glycoside for 1 h, and stimulated with LPS for 12 h. Total RNA from each group was isolated with TRIzol reagent. Approximately 5 μg of total RNA was used for RT using oligo-dT adaptor primer and superscript reverse transcriptase. PCR primers used in this study were purchased from Sangon Inc., (Shanghai, China) and are listed below:

COX-2 sense, 5'-TCTCCAACCTCTCCT-ACTAC-3'; COX-2 anti-sense, 5'-GCACGT-AGTCTTCGATCACT-3'; iNOS sense, 5'-CCCTTCCGAAGTTTCTGGCAGC-3'; iNOS anti-sense, 5'-GGCTGTCA GAGCCT-CGTGGCTT-3'; GAPDH sense, 5'-GACTT-CAACAGCAACTCCCACTC-3'; and GAPDH anti-sense primers, 5'-TAGCCGTATT-CATTGTCATACCAG-3'.

After amplification, PCR products were electrophoresed on 1.0 % agarose gels and visualized by ethidium bromide staining and UV irradiation.

Western blot analysis

RAW 264.7 cells placed in a 12-well plate were pretreated with various concentrations (20, 40, 80, and 160 μg/mL) of the flavone glycoside for 1 h, and stimulated with LPS for 6 h. After the incubation period, the cells were scraped from flasks and lysed in a lysis buffer (100 mL: 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1 M (v/v) SDS, 1% (v/v) Triton X-100, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstain). The samples were boiled at 100 °C for 5 min and centrifuged at 13,000 rpm for 2 min at 4 °C. Protein extracts were run on 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). The membranes were blocked with 5 % non-fat dry milk in TBS-T buffer for 1 h at room temperature. After blocking, the membranes were incubated with an appropriate dilution ratio of the relative primary antibody overnight at 4 °C. The membranes were incubated with secondary antibody for 4 h at room temperature, and detected by an ECL reagent.

Statistical analysis

Data are presented as means ± standard deviation (SD). For statistical analysis, data were analyzed by one-way ANOVA, followed by Duncan post-hoc pair-wise comparisons between groups. A significant difference between groups was set at $P < 0.05$.

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RESULTS

Cell viability

Figure 2 indicates the cell viability at 0, 20, 40, 80, 160, and 240 µg/mL selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside in the presence of LPS (100 ng/mL). After 24 h of incubation, the flavone glycoside at 240 µg/mL significantly decreased cell viability to about 90 % ($P < 0.05$), whereas the flavone glycoside at concentrations ranging from 10 µg/mL to 160 µg/mL did not exhibit any cytotoxic effect. Therefore, concentrations of flavonoids were selected from 20 µg/mL to 160 µg/mL for studies on the anti-inflammatory effect.

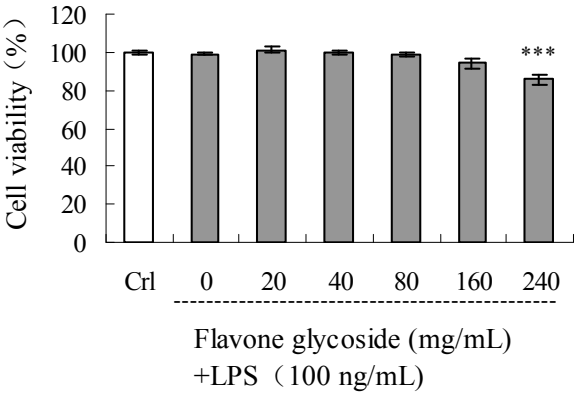

Figure 2: Growth inhibitory effect of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside in LPS-stimulated RAW 264.7 cells. Values are expressed as mean ± SD (*n* = 3). *** indicates $P < 0.001$ compared with the LPS only treatment group.

Effect of selagin-7-O-(6"-O-Acetyl-)-β-D-glucoside on LPS-induced inflammatory cytokines

Treatment of RAW 264.7 cells with LPS alone resulted in an increase in the release of all the pro- and anti-inflammatory cytokines analyzed (TNF-α, IL-1b, IL-6, and IL-10) compared with non-activated controls (Figure 3). Increased levels of pro-inflammatory cytokines IL-1β (Figure 3A), IL-6 (Figure 3B), and TNF-α (Figure 3C) in RAW 264.7 cells by LPS stimulation were dramatically reduced in a dose-dependent manner by exposure to the flavone glycoside ($P < 0.05$). At the same time, the level of anti-inflammatory cytokine, IL-10, significantly increased in a dose-dependent manner by exposure to the flavone glycoside ($P < 0.05$) (Figure 3D).

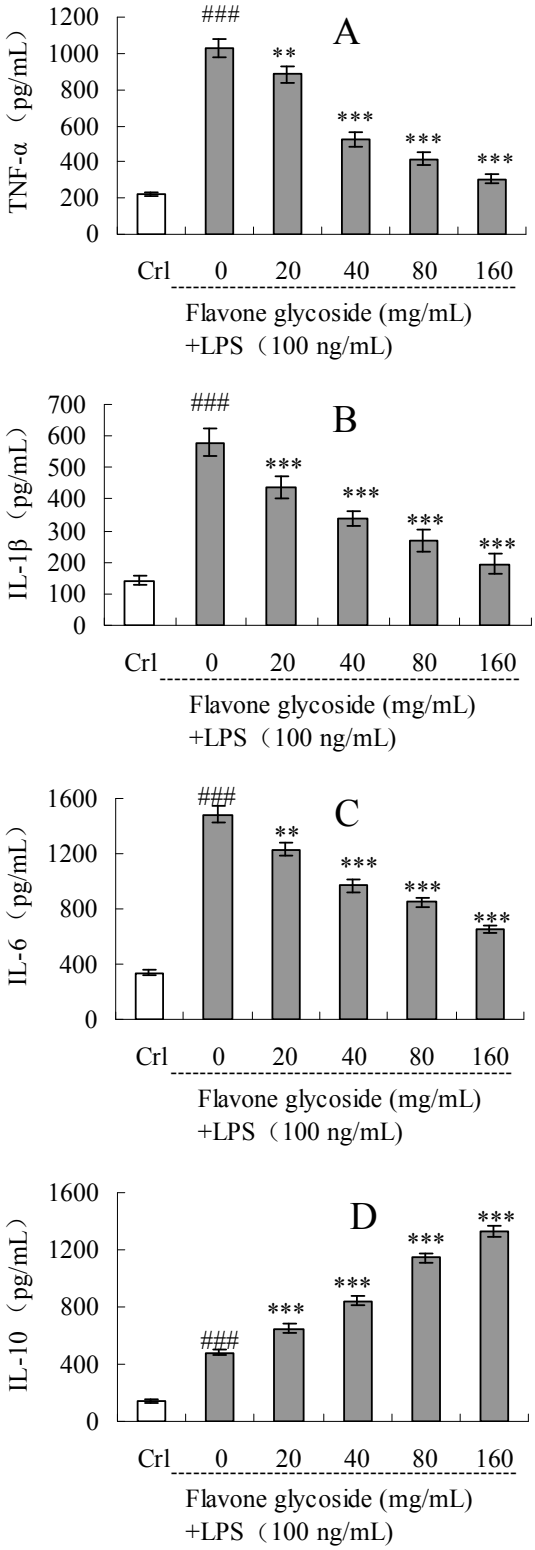

Figure 3: Effects of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside on LPS-induced TNF-α production of RAW 264.7 macrophages **(A).** Effects of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside on LPS-induced IL-1β production of RAW 264.7 macro-

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phages **(B).** Effects of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside on LPS-induced IL-6 production of RAW 264.7 macrophages **(C).** Effects of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside on LPS-induced IL-10 production of RAW 264.7 macrophages **(D).** Values are expressed as mean ± SD (*n* = 3). ### indicates P < 0.001 compared with the control group. *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, compared with the LPS only treatment group.

Effect of selagin-7-O-(6"-O-Acetyl-)-β-D-glucoside on LPS-induced NO and PGE2 production

NO and PGE2 production was examined in RAW 264.7 cells stimulated with LPS for 24 h in the presence or absence of the flavone glycoside. The levels of nitrite and PGE2 increased significantly in the culture medium. The flavone glycoside (20, 40, 80, and 160 μg/mL) markedly inhibited LPS-induced NO and PGE2 production in RAW 264.7 cells in a dose-dependent manner (Figures 4A and 4B).

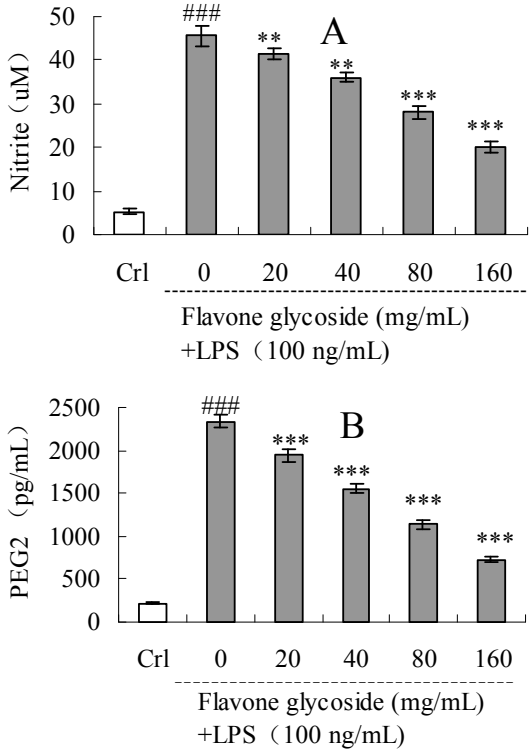

Figure 4: Effects of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside on LPS-induced NO production of RAW 264.7 macrophages **(A),** and effects of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside on LPS-induced PGE2 production of RAW 264.7 macrophages **(B)**. Values are expressed as mean ± SD (*n* = 3). ### indicates P < 0.001 compared with the control group. *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, compared with the LPS only treatment group.

Effect of selagin-7-O-(6"-O-Acetyl-)-β-D-glucoside on LPS-induced iNOS and COX-2 proteins and mRNA expression

The mRNA expression levels of COX-2 and iNOS significantly increased upon LPS treatment, and this induction was effectively inhibited in a dose-dependent manner by the flavone glycoside (Figures 5A and 5B). The protein expression levels of COX-2 and iNOS were similar. LPS-activated macrophages increased the expression of COX-2 and iNOS proteins compared with the untreated control group. However, the flavone glycoside prominently suppressed the LPS-induced COX-2 and iNOS protein expression in a dose-dependent manner (Figures 5C and 5D).

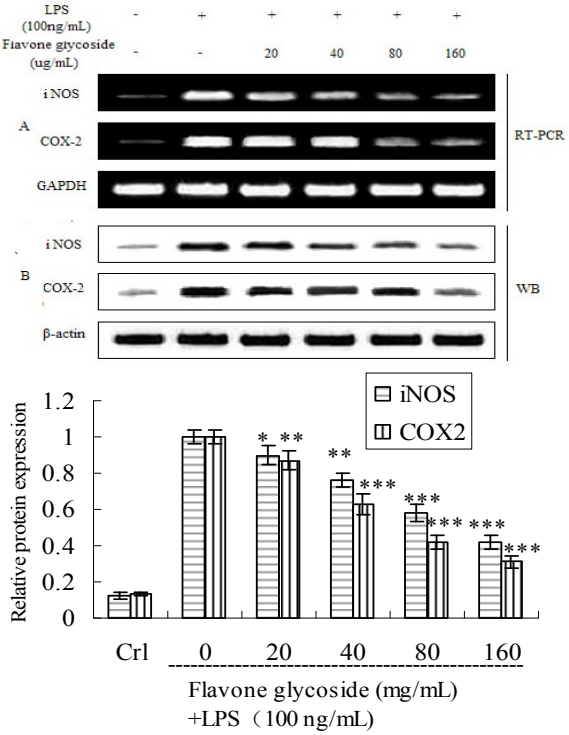

Figure 5: Inhibitory effect of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside on mRNA expression (A) and protein expression (B) of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. The results presented are representative of three independent experiments. Values are expressed as mean ± SD (n = 3). ### indicates P < 0.001 compared with the control group. *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively, compared with the LPS only treatment group.

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Effect of selagin-7-O-(6''-O-Acetyl-)-β-D-glucoside on LPS-induced NF-κB protein expression

The protein expression levels of NF-κB p65 significantly increased upon LPS treatment, and this induction was effectively inhibited in a dose-dependent manner by the flavone glycoside. However, the flavone glycoside prominently suppressed LPS-induced NF-κB p65 protein expression in a dose-dependent manner (Figure 6).

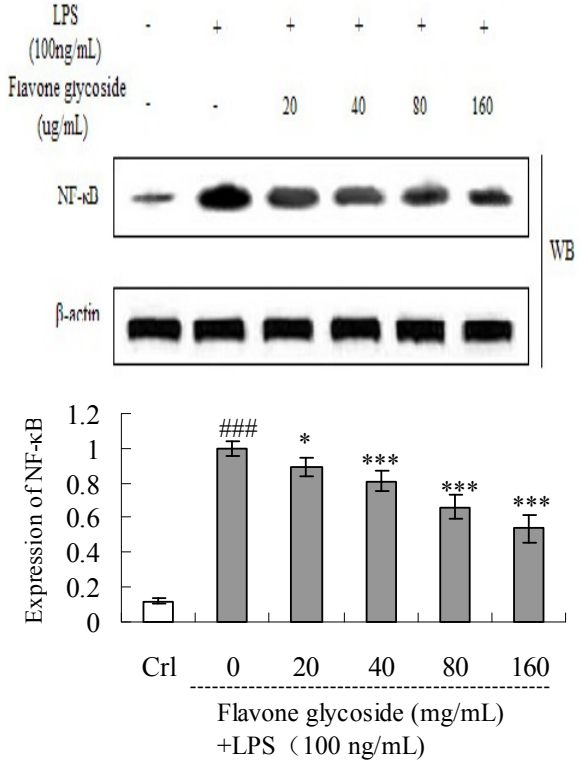

Figure 6: Inhibitory effect of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside on the expression of NF-κB in RAW 264.7 cells stimulated with LPS. Values are expressed as mean ± SD (n = 3). ### indicates $P < 0.001$ compared with the control group. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared with the LPS only treatment group.

DISCUSSION

Flavonoids, the group of low molecular weight polyphenolic compounds widely generated by plants and vegetables, exhibit various biological activities such as anti-oxidant, anti-inflammatory, anti-allergic, anti-microbial, anti-hypertensive, anti-cancer and anti-cancer activities (Chen et al., 2014; Nijveldt et al., 2001; Cotelle, 2001). In a previous study, selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside was shown to possess anti-inflammatory activity in rodent models (Su et al., 2011). However, the mechanisms underlying this anti-inflammatory activity remain unknown.

The anti-inflammatory properties of a wide variety of flavonoid molecules have been studied in deep detail, and several possible mechanisms of action have been explained. Free radicals play a critical role in inflammatory processes, so that radical scavenging effects of flavonoids are considered as the important mechanisms for anti-inflammatory activity (Soberón et al., 2010; Middleton et al., 2000; Lee, 2011). Some studies have shown that anti-inflammatory activity of flavonoids due to their ability to inhibit the arachidonic acid metabolism (Ferrandiz and Alcaraz 1991; Middleton et al., 2000; Di Carlo et al., 1999). In recent years, more and more research has shown that the mechanisms for anti-inflammatory activity of flavonoids are attributed to theirs ability to regulate the production of inflammatory mediators by inhibiting NF-κB activation (Lee, 2011; Ci et al., 2010; Kim et al., 2012; Kang et al., 2011).

In many studies, anti-inflammatory compounds have been investigated for their potential inhibitory effects in vitro using LPS-stimulated RAW 264.7 macrophages. Macrophages are important inflammatory cells involved in the initiation of inflammatory responses. During the inflammatory response, diverse mediators (e.g., NO, prostaglandins, and pro-inflammatory cytokines) are excessively produced by macrophages (Hu et al., 2011). LPS, an endotoxin derived from the cell wall of Gram-negative bacteria, has been referred to as a key risk factor associated with the inflammatory response, including septic shock, fever, and microbial invasion (Kim et al., 2010; Jung et al., 2007). An increasing amount of evidence has demonstrated that LPS can trigger the most potent microbial initiators of inflammatory responses by activating numerous inflammatory cells

(Tumurkhuu et al., 2008; Mirzapoiazova et al., 2007). Therefore, the anti-inflammatory activity of the flavone glycoside was further evaluated in the present study using in vitro inflammatory models to determine the exact molecular mechanisms behind this activity.

Pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, can contribute to tissue damage and multiple organ failure. TNF-α is involved in many different cellular processes, including production of numerous cytokines and acute phase proteins, thereby contributing to many pathophysiologic processes (Bradley, 2008). IL-1β, one of the most important inflammatory cytokines secreted by macrophages, increases in the release of IL-1β, which leads to cell or tissue damage (Molloy et al., 1993; West et al., 1995). IL-6 is also a pivotal pro-inflammatory cytokine that is regarded as an endogenous mediator of LPS-induced fever (Van Snick, 1990). IL-10 is the most important anti-inflammatory cytokine. In macrophages, IL-10 diminishes the production of inflammatory mediators and inhibits antigen presentation (Sabat et al., 2010). In the present study, we demonstrated that selagin-7-O-(6″-O-Acetyl-)-β-D-gluco-side inhibited TNF-α, IL-1β, and IL-6 production and increased IL-10 production in LPS-stimulated RAW 264.7 cells.

NO and PGE2 produced by activated macrophages have critical functions in inflammatory diseases. An overproduction of NO and PGE2, which are produced by iNOS and COX-2, can produce cytotoxic effects in pathological processes, especially in inflammatory and autoimmune disorders (Palmer et al. 1988); NO and PGE2 have been implicated as important mediators of inflammation. Therefore, a drug capable of preventing the release of pro-inflammatory mediators in inflammatory cells may possess anti-inflammatory activities. In the present study, the flavone glycoside inhibited the production of NO and PGE2 in LPS-induced RAW 264.7 cells, which was associated with the down-regulation of iNOS and COX-2 genes.

NF-κB is an essential transcription factor regulating the expression of inflammation-induced enzymes and cytokines, such as iNOS, COX-2, TNF-α, and IL-6 (Lawrence et al., 2001). Under normal conditions, NF-κB exists as a heterodimer consisting of p50 and p65 and is bound to an inhibitor of kappa B in the cytoplasm (Karin, 1999). Signal cascades triggered by pro-inflammatory cytokines, such as TNF-α and IL-1β, bacterial endotoxin (e.g., LPS), and oxidative stress can activate IκB kinases. The NF-κB complex is free to enter the nucleus, which leads to the transcription of target genes, such as the pro-inflammatory mediators COX-2 and iNOS; various cytokines, including TNF-α, IL-1β, and IL-6; chemokines, and adhesion molecules (Lawrence et al., 2001). The present results show that the flavone glycoside significantly inhibited the LPS-induced activation and nuclear translocation of NF-κB p65 at various concentrations.

CONCLUSION

Taken together, the in vitro studies using LPS-stimulated RAW 264.7 macrophage models demonstrate for the first time that the mechanisms underlying the anti-inflammatory activity of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside dependent on its ability to regulate the production of inflammatory mediators and cytokines, via blocking activation of NF-κB. The results of in vitro and in vivo studies provide scientific supporting evidence that flavonoids may be the active constituents related to the traditional use of *C. discoidea* in some painful and inflammatory conditions. However, it limits the research about the flavone glycoside that selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside is just one of flavones isolated from *C. discoidea*, and it is rare in the plant. Future studies will be necessary in order (1) to assess the in vivo toxicity of selagin-7-O-(6″-O-Acetyl-)-β-D-glucoside, (2) to determine the bioavailability and metabolic mechanism of the flavone glycoside, (3) to determine the long term stability of the flavone glycoside after storage under an appropriate form.

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