TANG [HUMANITAS MEDICINE]

Original Article

Anti-oxidant and anti-inflammatory effects of *Salix Koreensis* Andersson in DC. leaf methanol extract *in vitro* models

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ABSTRACT

Oxidative rancidity in foods causes undesirable changes in nutritive value, aroma, flavor, and color. *Salix Koreensis Andersson* in DC. (SK) has anti-oxidative and anti-inflammatory effects and is traditionally used to treat neuralgia, edema, pain, and inflammatory diseases. However, the regulatory effects of SK on oxidative and inflammatory reactions have not been elucidated. In this context, we scientifically validated the anti-oxidative and anti-inflammatory activities of SK leaf (SKL). The methanol extract of SKL was evaluated for *in vitro* anti-oxidative activities. SKL showed increased superoxide dismutase (SOD)-like activity and 1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity. The in vitro anti-oxidant and anti-inflammatory activities of SKL were also investigated in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. LPS resulted in decreased SOD activities compared with the unstimulated cells, but SKL significantly increased SOD activities reduced by LPS. In addition, LPS-induced nitric oxide, tumor necrosis factor-α, and interleukin-6 productions were significantly and dose-dependently reduced by SKL in RAW264.7 macrophages without inducing cytotoxicity. In conclusion, these results indicate that SKL will be able to be effectively used as a food additive with anti-oxidative and anti-inflammatory effects.

Keywords *Salix Koreensis* Andersson in DC., anti-oxidant activity, anti-inflammatory activity, superoxide dismutase

INTRODUCTION

Oxidation is the key process for production of energy in an organism to fuel biological processes (Kong et al., 2010). However, excessively generated free radicals free radicals increase the oxidative stress in organisms by causing deleterious effects to cell structures. Many disease conditions like diabetes, inflammation, cancer, atherosclerosis, liver diseases, aging, hypertension, and neurodegenerative diseases precipitate directly or indirectly through reactive oxygen species (ROS)-mediated signal pathways (Liang et al., 2011; Sarwar et al., 2015). Furthermore, oxidative rancidity in foods leads to reduction in its shelf life. Oxidative degradation causes undesirable changes of color, nutritive value, aroma, and flavor which eventually affect consumer approval rating (Nerin et al., 2008). Antioxidants neutralize the free radicles generated in the body and activate superoxide dismutase (SOD, one of the major antioxidant enzymes) activity and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (Fu et al., 2010). Synthetic antioxidants are often used in foods to block oxidative deterioration (Pawar et al., 2012). Though synthetic antioxidants are widely used yet it is suspected to induce tumor formation in animals (Clayson et al., 1993; Hocman, 1988). Oxidative stress also generate inflammatory cascades, which are primarily mediated via inflammatory molecules including nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin (IL)-6 (Sun et al., 2016). During inflammation processes, NO, TNF-α, and IL-6 were produced from activated macrophages (Kitanaka, 2016). Thus, inhibition of these inflammatory mediators is regarded as an attractive therapeutic strategy for inflammatory diseases.

Salix species have been used to treat pain, inflammation, and fever since ancient times (Du et al., 2007; Freischmidt et al., 2012). Moreover, Salix species also have anti-oxidant (Alam et al., 2006), anti-inflammatory (Li et al., 2008), anti-obesity (Han et al., 2003), and anti-tumor (Sultana et al., 2004) effects. *Salix Koreensis Andersson* in DC. (Korean weeping willow, SK) is a valuable tree that could be used as a potential tree for phytoremediation. Salix Koreensis stem extract showed the anti-elastase and anti-tyrosinase activities (Park et al., 2007).

Oxidative stress is tightly connected with inflammatory reactions (Sun et al., 2016). SK has anti-oxidative and anti-inflammatory effects and is traditionally used to treat neuralgia, edema, pain, and inflammatory diseases in Korea. However, the regulatory effects of SK on oxidative and inflammatory reactions have not been elucidated. Keeping in view the increasing demand for natural additives, the present study evaluated the effect of SK leaf (SKL) as a food additive on oxidative and inflammatory reactions using *in vitro* assays and RAW264.7 macrophages.

MATERIALS AND METHODS

Meterials

Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-

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Table 1. Effect of SKL on SOD-like activity and DPPH radical scavenging activity

Sample	SOD-Like Activity (%)	EDA (%)
SKL (1 μg/ml)	82.53 ± 1.61	79.26 ± 0.73
***p*-value**	0.002*	0.0001*

Data are mean SEM values of three independent experiments performed in duplicate. EDA, Electron donating ability; SKL, Leaf of Salix Koreensis Andersson in DC.

diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and sodium nitrite were purchased from Sigma Chemical Co. (St Louis, MO, USA). Murine recombinant (r)TNF-α and rIL-6, anti-mouse TNF-α and IL-6, and biotinylated mouse TNF-α and IL-6, were purchased from BD Pharmingen (San Diego, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) containing L-arginine (84 mg/l), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL (Grand Island, New York, USA).

Preparation of SKL

SKL was prepared by methanol extraction. The SKL were extracted for 1 h with 7.5 ml of a methanol: acetone: water solution (1:1:1; v/v/v). The extracts were centrifuged (6800 × g, 30 min) and extraction procedure was repeated two times. The crude extracts were filtered and concentrated in vacuo at 60℃. It was lyophilized. A voucher specimen (No.20160601) was deposited in Department of Food and Nutrition, Hoseo University. Total phenolic acids were measured as previously described by Benvenuti et al. (2004). Total flavonoids content in SKL was determined by the modified Davis method (1947). SK contained about 3.91% phenolic acids and about 2.23% flavonoids. The SKL was prepared by dissolving it in methanol at 10 mg/ml and then diluted with phosphate-buffered saline (PBS).

Assay for SOD-like activity

SOD-like activity was evaluated by modified SOD-like assay (Tsuda et al., 1995). The reaction solution was prepared by mixing 0.2 ml of SKL, 3 ml of tris-HCl buffer (pH 8.5), and 0.2 ml of 7.2 mM pyrogallol, which was then placed at 25℃ for 10 min. The oxidized pyrogallol was measured at 420 nm using an ELISA reader (Molecular Devices Corp., Sunnyvale, California, USA) after the reaction was stopped by adding 0.1 ml of 1.0 N HCl. The SOD-like activity is expressed as the reduction rate of absorbance.

SOD-like activity (%) = [1−(absorbance value of testing solution/absorbance value of control solution)] × 100

DPPH radical scavenging assay

The radical scavenging activity was evaluated by modified DPPH assay (Kang et al., 2001). Briefly, to 3.5 ml of 0.15 mM DPPH, 0.5 ml of the SKL extract solution (1 μg/ml) was added. The mixture was shaken vigorously and incubated at room temperature for 30 min. Next, the mixed samples were measured using an ELISA reader (Molecular Devices Corp., Sunnyvale, California, USA) at 517 nm. The electron donating ability (EDA) was calculated as follows:

EDA (%) = [1−(absorbance value of testing solution/absorbance value of control solution)] × 100

Cell culture

RAW264.7 macrophages were grown in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat inactivated FBS at 37℃, 5% CO_2 and 95% humidity. Cells were pretreated with SKL for 1 h prior to LPS stimulation. Methanol (0.1%) was treated as a negative control.

Measurement of SOD activities

RAW264.7 macrophages (1×10^6 cells/well) were pretreated with SKL for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. Cells were then lysed by addition of 50 μl lysis buffer, and the homogenate was centrifuged at 12000 rpm for 10 min at 4℃. The obtained supernatants were then used for antioxidant enzyme activities. The SOD activity was determined by following the manufacturer's protocol using SOD determination kit purchased from abcam (Cambridge, UK).

Measurement of nitrite concentration

RAW 264.7 macrophages (1×10^5 cells/well) were pretreated with SKL for 1 h and then stimulated with LPS (1 μg/ml) for 48 h. To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined using an ELISA reader (Molecular Devices Corp., Sunnyvale, California, USA). NO_2^- was determined by using sodium nitrite as a standard.

MTT assay

RAW264.7 macrophages (1×10^5 cells/well) were cultured for 24 h with SKL. Cell aliquots were incubated with 40 μl of a MTT solution (5 mg/ml) for 4 h at 37℃ under 5% CO_2 and 95% air. Consecutively, 500 μl of DMSO was added to extract the MTT formazan and the absorbance of each well was read using an ELISA reader at 540 nm (Molecular Devices Corp., Sunnyvale, California, USA).

Assay of cytokine release

RAW264.7 macrophages (1×10^5 cells/well) were pretreated with SKL for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. The amounts of TNF-α and IL-6 secreted from RAW264.7 macrophages were measured by a modified

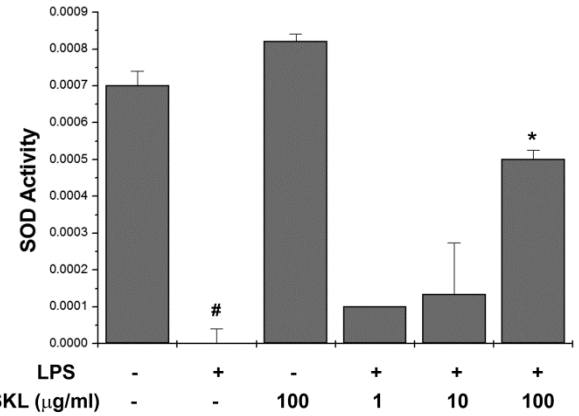

Fig. 1. Effects of SKL on SOD activation. Cells were pretreated with SKL (1, 10, 100 μg/ml) for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. The levels of SOD were measured by SOD assay kit. Data are mean ± SEM values of three independent experiments performed in duplicate. $^{\#}p < 0.05$: significantly different from the unstimulated cells (0.1% methanol); $^{*}p < 0.05$: significantly different from the LPS-stimulated cells. LPS, lipopolysaccharide; SKL, Leaf of *Salix Koreensis* Andersson in DC.

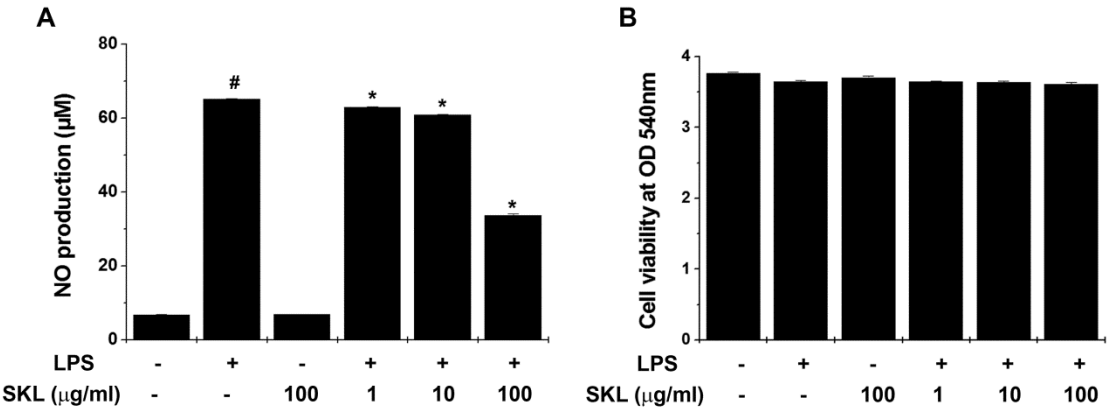

Fig. 2. Effects of SKL on LPS-induced NO production and cell viability. (A) Cells were pretreated with SKL (1, 10, 100 μg/ml) for 1 h and then stimulated with LPS (1 μg/ml) for 48 h. NO production was measured by Griess method. (B) Cells were pretreated with SKL (1, 10, 100 μg/ml) for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. Cell viability was determined with an MTT assay. Data are mean ± SEM values of three independent experiments performed in duplicate. $^{\#}p < 0.05$: significantly different from the unstimulated cells (0.1% methanol); $^{*}p < 0.05$: significantly different from the LPS-stimulated cells. LPS, lipopolysaccharide; SKL, Leaf of Salix Koreensis Andersson in DC.

enzyme-linked immunosorbent assay (ELISA). The ELISA was devised by coating 96-well plates of murine monoclonal antibodies (1 μg/ml) with specificity for TNF-α and IL-6. Before use and between subsequent steps in the assay, coated plates were washed with PBS containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37℃. The rTNF-α and rIL-6 were diluted and used as a standard. Serial dilutions starting from 10 ng/ml were used to establish the standard curve. After 4 h incubation at 37℃, the wells were washed and then each of 1 μg/ml of biotinylated anti-mouse TNF-α and IL-6 were added and the plates were incubated at 37℃ for 45 min. After washing the wells, avidin-peroxidase was added and the plates were incubated for 30 min at 37℃. Wells were again washed and substrate solution was added. Color development was measured at 405 nm using an ELISA reader (Molecular Devices Corp., Sunnyvale, California, USA).

Statistical analysis

The data are presented as means ± standard error of means (SEMs) of three independent experiments performed in duplicate. Statistical evaluation of the results was performed by an independent *t*-test and ANOVA with Tukey's post hoc or Dunnett T3 test. All statistical analyses were performed using SPSS v12.00 statistical analysis software (SPSS Inc.). The results were considered significant at a value of $P < 0.05$.

RESULTS

Effect of SKL on SOD-like activity and DPPH radical scavenging activity

Oxidative reaction is induced and exaggerated by an imbalance the activities of ROS and antioxidant molecules and excessive ROS production causes various diseases (Kandimalla et al., 2016; Sarwar et al., 2015). To investigate the anti-oxidative activity of SKL, we measured SOD-like activity. As a result, SKL had a SOD-like activity of 82.53% at 1 μg/ml (Table 1). Next, the EDA of SKL at 30 min was measured. SKL showed DPPH radical scavenging activity (about 79%, Table 1).

Effects of SKL on LPS-induced SOD activation

To evaluate the protective function of SKL against oxidative stress, the activities of SOD were measured in the LPS-treated RAW 264.7 macrophages. LPS caused a significant decrease in SOD activities compared with the untreated cells (Fig. 1, $p < 0.05$). However, the SOD activities reduced by LPS were dose-dependently increased by SKL (Fig. 1, $p < 0.05$). Methanol (0.1%) did not affect SOD activity as compared with untreated cells.

Effects of SKL on LPS-induced NO production

The high amount of NO produced following exposure to LPS plays a vital role in increasing inflammatory reactions (Jeong et al., 2014). To examine the effect of SKL on LPS-induced NO production, we carried out Griess reagent method. As shown in Fig. 2A, the NO production was significantly elevated in cells with LPS alone ($p < 0.05$). However, the LPS-induced NO production was significantly suppressed by treatment with SKL ($p < 0.05$). Cytotoxic effect of SKL on RAW264.7 macrophages were evaluated by MTT colorimetric assay. As shown in Fig. 2B, cell viabilities were not affected by SKL in the RAW264.7 macrophages.

Effects of SKL on LPS-induced proinflammatory cytokine production

Macrophages play a central role in inflammatory reactions (Lawrence et al., 2002). When macrophages are stimulated with LPS, the production of pro-inflammatory cytokines is markedly increased (Jeong et al., 2014). To study the effect of SKL on the production of proinflammatory cytokines, we performed ELISA. Compared with the untreated cells, cells treated with LPS alone significantly promoted the production of TNF-α and IL-6. Meanwhile, LPS-induced TNF-α and IL-6 productions were significantly attenuated by SKL (Fig. 3, $p < 0.05$).

DISCUSSION

In the present study, we found that SKL has an anti-oxidative effect via increasing the SOD and DPPH radical scavenging activities. In addition, SKL showed the anti-inflammatory effects through decreasing the NO and inflammatory cytokines production.

Oxidative stress is known to influence the activities of inflammatory mediators and other cellular signaling pathways involved in the initiation, promotion, and progression of many diseases. Oxidation also induced nutritional and functional

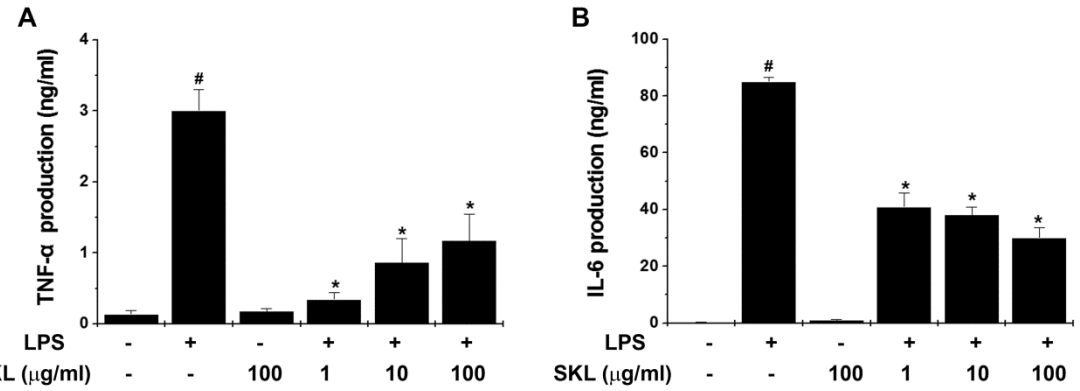

Fig. 3. Effects of SKL on LPS-induced TNF-α and IL-6 production. Cells were pretreated with SKL (1, 10, 100 μg/ml) for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. The levels of (A) TNF-α and (B) IL-6 were measured by ELISA method. Data are mean ± SEM values of three independent experiments performed in duplicate. $^{\#}p < 0.05$: significantly different from the unstimulated cells (0.1% methanol); $^{*}p < 0.05$: significantly different from the LPS-stimulated cells. LPS, lipopolysaccharide; SKL, Leaf of Salix Koreensis Andersson in DC.

degradation of foods. Antioxidants were used to prevent the oxidative rancidity of foods through the activation of SOD (Sęczyk et al., 2017; Siwach et al., 2016). Snthetic antioxidants were also often used but most of these antidepressants have several adverse side effects. Consumers' awareness of the health risks of synthetic antioxidants and demand for natural food ingredients has resulted in extensive research on naturally occurring antioxidants (Dua et al., 2015; Iqbal et al., 2007; Sivam et al., 2010). SK contains substantial amounts of phenolic acids, such as, coumaric aicd, caffeic acid, ferulic acid, salicin, epicathechin, tannin, flavonoid, and luteolin. Especially, aspirin (acetylsalicylic acid, ASA) is used to reduce fever and relieve mild to moderate pain from conditions such as muscle aches, toothaches, common cold, and headaches. Additionally, the phenolic compounds are known for their antioxidant activity. Phenolics as natural antioxidants preserve human body from oxidative stress, which is a major factor increasing the incidence of inflammatory diseases such as inflammatory, cancer, Alzheimer's disease, and cardiovascular (Carocho et al., 2013; Scalbert et al., 2005). In the present study, we showed that SKL increased the SOD-like activity and DPPH radical scavenging activity. Therefore, we presupposed that SKL is an antioxidant and phenolics are active compounds of SKL. However, further studies are necessary to determine the isolation of active ingredient of SKL and underlying mechanisms of active ingredient.

Inflammation is an acute response triggered by noxious stimuli and tissue damage with classical signs of acute inflammation, such as swelling, pain, redness, and heat (Ricciotti and FitzGerald, 2011). Acute inflammation is the initial response to harmful stimuli and is achieved by the elevated movement of plasma and granulocytes such as macrophages, eosinophils, basophils, and neutrophils (Shpacovitch et al., 2007). Macrophages play major roles in inflammation, immunity and host defense mechanisms. Once activated, they produce and release NO, inflammatory cytokines, and eicosanoids (Kitanaka, 2016). NO is a central inflammatory molecule and produced by inducible nitric oxide synthase under various physio-pathological conditions. Over-produced NO causes accumulation of ROS, disruption of tissue homeostasis, and cell death (Pando et al. 2000). Thus, NO was used as a biomarker for inflammation (Hosseini et al. 2006). Inflammatory cytokines such as TNF-α and IL-6, and are identified as key players in leukocyte infiltration into the inflamed tissues, which causes damage (Soufli et al., 2016). The macrophages stimulated by LPS secret TNF-α and IL-6 (Medzhitov et al., 1997). TNF-α resulted in leukocyte infiltration, granuloma formation, inflammation, and tissue fibrosis and accelerated inflammatory response through increasing inflammatory cytokine production (Balkwill, 2009). Kennedy-Feitosa et al. (2016) reported that TNF-α receptor deficient mice do not develop inflammation, suggesting a key role of TNF-α in response to inflammatory disease. IL-6 is also a major cytokine that is responsible for the initiation and propagation of inflammation by mediating the acute phase and can be stimulated by TNF-α (Basinska et al., 2015; Kitanaka, 2016). Therefore, suppression of macrophage activation is correlated with inhibition of inflammatory diseases. Freischmidt et al. (2012) reported that flavonoids and catechol isolated from SK bark extract has an anti-inflammatory effect. In this study, we showed for the first time that SKL suppresses the LPS-induced NO, TNF-α and IL-6 production. Therefore, these results indicate that SKL exhibits anti-inflammatory activities in LPS-stimulated macrophages.

In the Fig 3A, the lowest concentration of SKL seems to be most effective. However, SKL inhibited the SOD, NO, and IL-6 in a dose-dependent manner. Therefore, further investigation is required to clarify more precisely regulatory effect of SKL on the activation of SOD and production of NO and inflammatory cytokines.

In conclusion, we demonstrated that SKL increased the SOD-like activity and DPPH radical scavenging activity. In addition, SKL significantly increased the SOD activity and significantly decreased the NO, TNF-α, and IL-6 production on LPS-stimulated RAW264.7 cells. Therefore, these results demonstrate that SKL may be a beneficial food additive with anti-oxidative and anti-inflammatory effects.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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