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NOTE

Anti-inflammatory effect of *Rhus verniciflua* stokes extract in the murine macrophage cell line, Raw264.7

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Abstract *Rhus verniciflua* stokes (RVS) (Anacardiaceae), which contains the major flavonoids fustin, fisetin, and sulfuretin, is known to have anti-inflammatory effects. Using lipopolysaccharide (LPS)—induced RAW264.7 cells, we examined which flavonoids were the most active compounds in the RVS extract. RVS extract dose-dependently reduced the production of nitric oxide, prostaglandin E_2, interleukin-6 (IL-6), and reactive oxygen species (ROS) induced by LPS. Only sulfuretin significantly suppressed IL-6 and ROS levels although the effects were smaller than those provided by the RVS extract at the equivalent concentration (0.25 μg/mL of pure sulfuretin and 100 μg/mL of RVS extract). Other flavonoids such as fisetin and fustin did not show anti-inflammatory effects at the levels typically contained in the RVS extract. Taken together, sulfuretin was postulated to be the major anti-inflammatory flavonoid in RVS extract.

Keywords Inflammation · Lipopolysaccharide · *Rhus verniciflua* stokes extract · Sulfuretin

Introduction

Rhus verniciflua stokes (RVS) (Anacardiaceae) is one of the main medicinal edible plants in Korea, Japan, and China and is used as a folk remedy for gastritis, several cancers, and various metabolic diseases and as a foodstuff (Choi et al. 2012). Furthermore, the stem bark of RVS has been reported to have various pharmacological effects including antitumor, antioxidant, and anti-aromatase activities (Lim et al. 2001; Lee et al. 2004; Jung et al. 2006; Park et al. 2014). Of particular interest is its anti-inflammatory activity (Lee et al. 2010; Shin et al. 2010; Choi et al. 2014a). Lee et al. reported that sulfuretin was the major flavonoid in RVS, and reduced the inflammatory response through iNOS and COX-2 suppression (Lee et al. 2010). However, in our previous studies, fustin was the most abundant flavonoid in RVS and thus, we standardized the RVS extract based on its fustin concentration (Choi et al. 2014b). Besides fustin and sulfuretin, fisetin has been also known to alleviate inflammatory responses (Kitts and Lim 2001; Son et al. 2005; Jung et al. 2007). Plants contain a large array of active compounds and diverse arrays of bioactive components likely provide increased health benefits via synergistic effects (Kim and Kwon 2011). To further explore the beneficial properties of RVS extract, it is necessary to identify the most active flavonoid as well as the concentrations of the individual flavonoids. Therefore, in this study, we assessed the anti-inflammatory effect of RVS extract, hypothesizing that RVS might contain active flavonoids such as fustin, fisetin, and sulfuretin. By comparing the anti-inflammatory effect of RVS extract with single flavonoids, it may clarify which flavonoid is the most active.

Materials and methods

Chemicals and reagents

Dulbecco's modified eagle's medium (DMEM) was obtained from Welgene (Daegu, Korea). Fetal bovine serum (FBS) was obtained from Corning cellgro (Oneonta, New York, USA). Penicillin–streptomycin and HEPES were

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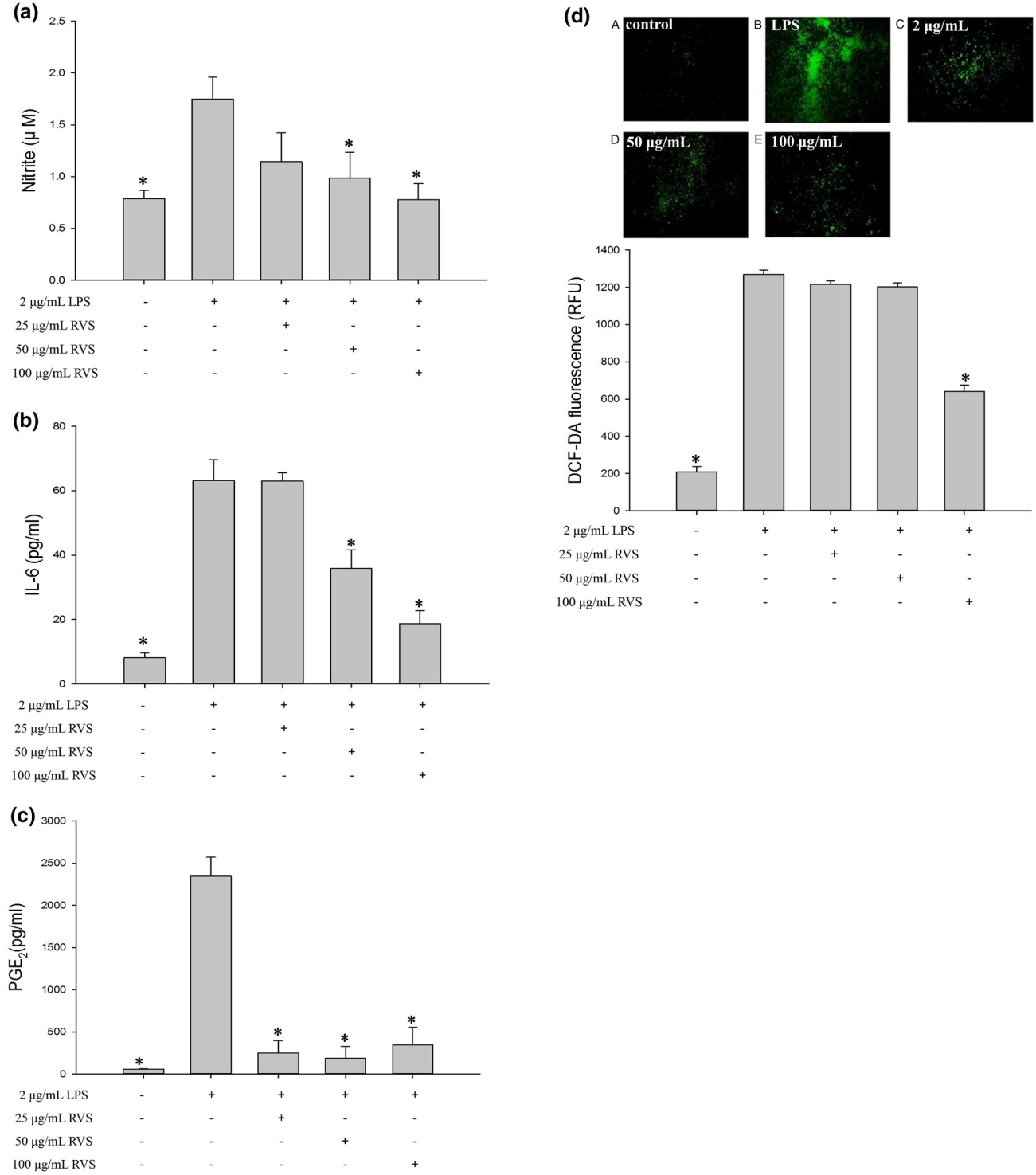

Fig. 1 Anti-inflammatory effects of RVS extract. NO production (**a**), IL-6 (**b**), PGE_2 (**c**), and ROS (**d**) were quantified using LPS-stimulated RAW264.7 cells. *Significant differences from the LPS-treated cells by ANOVA followed by Dunnett's test

obtained from Gibco (Rockville, MD, USA). LPS from *Escherichia coli* serotype 055:B5 (L4391), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, sulfanilamide, phosphoric acid, naphthylethylenediamine, dihydrochloride, and fisetin were obtained from Sigma-Aldrich (St. Louis, MO, USA).

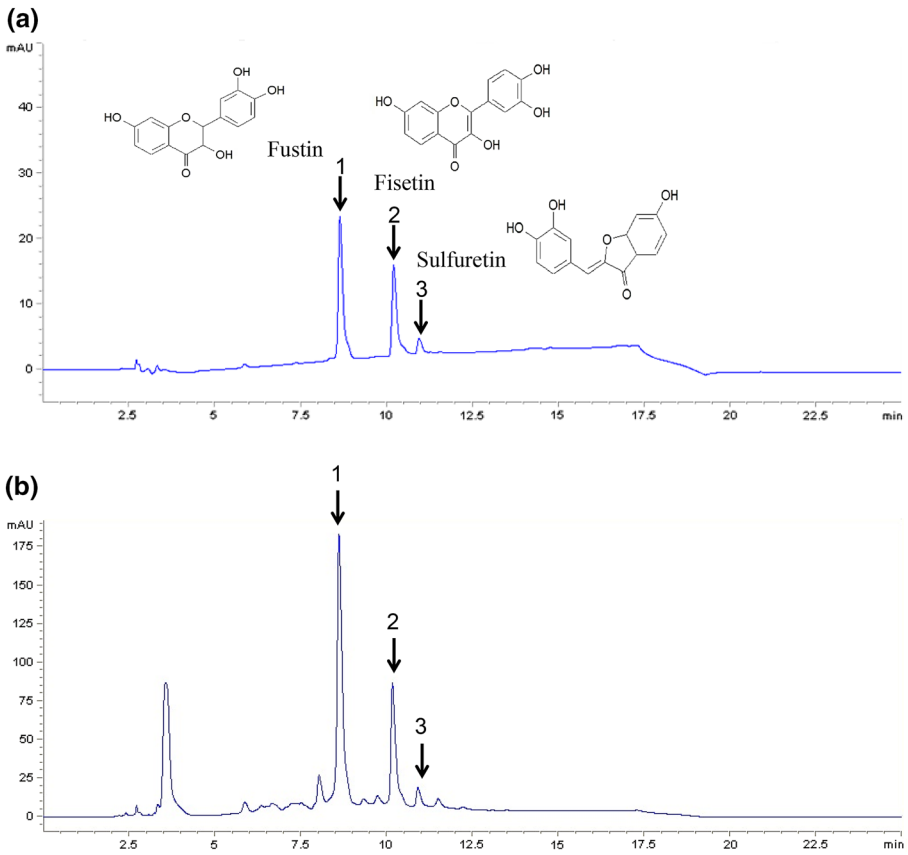

Fig. 2 Representative HPLC chromatograms of a standard mixture (**a**) and RVS extract (**b**). Peak 1 was identified as fustin, peak 2 was fisetin, and peak 3 was sulfuretin

Preparation of standardized *Rhus verniciflua* stokes (RVS) extract

The RVS extract standardized with 3 % fustin was kindly provided by Lifetree Biotech Co., Ltd. (Suwon, Korea). Briefly, dried branches of RVS collected from Kangwon Province (Korea) were cut into chips with a chip maker. The chips were added to water and extracted at 90–110 °C for 4 h. The extract was concentrated and treated with alcohol. The alcohol-treated extract was then freeze-dried. Fustin and sulfuretin standards were also provided from Lifetree Biotech Co., Ltd.

High-performance liquid chromatography

Preliminary chemical analysis of RVS extract was carried out using high-performance liquid chromatography (HPLC). The HPLC system consisted of a 1260 infinity HPLC system with a 1260 quaternary pump (Agilent Technologies, Palo Alto, CA). The chromatographic separation of the compounds was achieved using a Capcell Pak C_{18} (4.6 mm I.D. × 150 mm, 3 μm, Shiseido, Tokyo, Japan) with the column oven temperature maintained at 30 °C. The mobile phase consisted of 0.1 % formic acid (solvent A) and 90 % acetonitrile containing 0.1 % formic acid (solvent B). The mobile phase flow rate was 1 mL/min with gradient elution: 0–1 min, 10 %; 1–15 min, 80 %; 15–16 min, 10 %; 16–25 min, 10 % of solvent B. The injection volume was 10 μL, and the UV detection wavelength was set at 260 nm.

Measurement of nitric oxide, pro-inflammatory cytokines, and prostaglandin E_2 production

RAW264.7 cells (Korean Cell Line Bank, Seoul, Korea) maintained in DMEM medium containing 10 % FBS, 2 % penicillin–streptomycin, and 2 % HEPES at 37 °C in a humidified incubator (5 % CO_2 and 95 % air). Cells were incubated with a pre-treated different sample for 24 h and then stimulated with LPS (2 μg/mL) for 48 h. Nitric oxide (NO) production was quantified using the Griess reagent (0.1 % naphthylenediamine and 1 % sulfanilamide in 5 % phosphoric acid). Interleukin-6 (IL-6, USCN Life Science Inc., Wuhan, Hubei, China) and prostaglandin E_2 (PGE_2, Abcam, Cambridge, Massachusetts, USA) were analyzed using ELISA Kits as described in the supplier manuals.

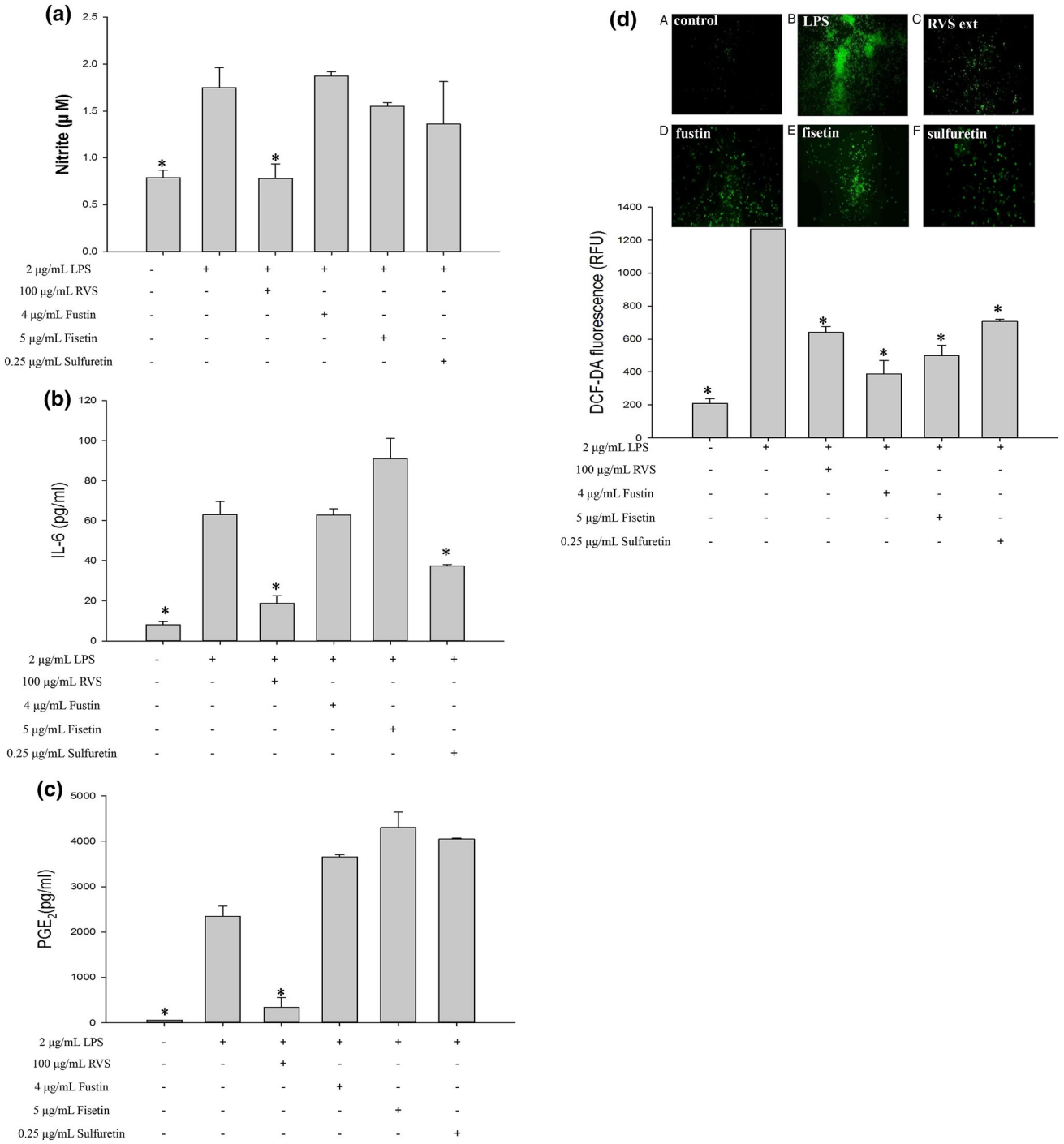

Fig. 3 Anti-inflammatory effects of RVS extract and its major flavonoids fustin, fisetin, and sulfuretin. NO production (**a**), IL-6 (**b**), PGE_2 (**c**), and ROS (**d**) were quantified using LPS-stimulated RAW264.7 cells. *Significant differences from the LPS-treated cells by ANOVA followed by Dunnett's test

Measurement of intracellular reactive oxygen species (ROS)

After culture and stimulation, cells were washed with DPBS and incubated for 30 min with dichlorofluorescein diacetate (DCF-DA) dissolved in DMSO (final concentration at 50 μM). Fluorescence was measured using a fluorescent microscope (Motic, Richmond, Canada) and spectrophotometer (PerkinElmer Inc., Waltham, MA, USA) at 480 nm excitation and 530 nm emission.

Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Comparisons between groups were performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

To confirm the effect of RVS extract on the suppression of inflammatory responses simulated with LPS (2 μg/mL) for 18 h in RAW264.7 cells, the levels of nitrite, pro-inflammatory cytokines, and PGE_2 were measured. As shown in Fig. 1, LPS increased inflammatory responses, whereas pretreating cells with RVS extract (25, 50 and 100 μg/mL) markedly reduced LPS-induced NO, IL-6, PGE_2, and ROS levels (Fig. 1). In this preparation, flavonoids such as fisetin, fustin, and sulfuretin were found to contain at concentrations of 53.59, 43.08, and 2.74 mg/g, respectively (Fig. 2). In this study, to examine which flavonoids contained in the RVS extract suppressed LPS-induced inflammation, RAW264.7 cells were pre-incubated with 4 μg/mL fustin, 5 μg/mL fisetin, 0.25 μg/mL sulfuretin, and 100 μg/mL RVS extract. As shown in Fig. 3, when the RAW264.7 cell line was treated with the flavonoids and the RVS extract, the levels of IL-6 were only significantly inhibited by sulfuretin, although the RVS extract suppressed IL-6 2 times more than sulfuretin (Fig. 3c). Fisetin and fustin did not show anti-inflammatory effects at the treatment concentrations. For purposes of comparison, the level of sulfuretin used in the present study was almost 1 μM, while the previous studies used 30 μM sulfuretin (Shin et al. 2010; Lee et al. 2012).

Fisetin and fustin have been reported to have anti-inflammatory effects. Recently, fisetin was found to inhibit UVB-induced cutaneous inflammation and high-glucose-induced vascular inflammation (Kwak et al. 2014; Pal et al. 2015). Fustin is one of the major flavonoids in *Toxicodendron vernicifluum* reported to have cytotoxic and anti-inflammatory effects, while sulfuretin showed the strongest NO suppressive effect in LPS-activated BV-2 cells (Kim et al. 2015). Therefore, based on the previous reports as well as our findings, although sulfuretin was the most active flavonoid in RVS extract, fisetin, fustin, or another flavonoid might contribute to suppressing inflammatory components and ROS.

The limitation of the present study is that we could not identify all the possible flavonoids in the RVS extract and could not confirm the effect of combining sulfuretin, fustin, and fisetin. Nonetheless, we did confirm that RVS showed a multi-component effect. Future work will include the identification of all the possible active flavonoid profiles of RVS extract and elucidation of their additive and synergistic effects.

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References

Choi W, Jung H, Kim K, Lee S, Yoon S, Park J, Kim S, Cheon S, Eo W, Lee S (2012) *Rhus verniciflua* stokes against advanced cancer: a perspective from the Korean Integrative Cancer Center. J Biomed Biotechnol 2012:874276

Choi HS, Seo HS, Kim SR, Choi YK, Jang BH, Shin YC, Ko SG (2014a) Anti-inflammatory and anti-proliferative effects of Rhus verniciflua Stokes in RAW264.7 cells. Mol Med Rep 9:311–315

Choi Y, Do G, Shin J, Kim J, Kwon O (2014b) Standardized *Rhus verniciflua* stokes extract and its major flavonoid fustin attenuate oxidative stress induced by tert-butyl hydroperoxide via activation of nuclear factor erythroid 2-related factor. J Korean Soc Appl Biol Chem 57:4

Jung CH, Jun C-Y, Lee S, Park C-H, Cho K, Ko S-G (2006) *Rhus verniciflua* stokes extract: radical scavenging activities and protective effects on H_2O_2-induced cytotoxicity in macrophage RAW 264.7 cell lines. Biol Pharm Bull 29:1603–1607

Jung CH, Kim JH, Hong MH, Seog HM, Oh SH, Lee PJ, Kim GJ, Kim HM, Um JY, Ko SG (2007) Phenolic-rich fraction from *Rhus verniciflua* stokes (RVS) suppress inflammatory response via NF-kappaB and JNK pathway in lipopolysaccharide-induced RAW 264.7 macrophages. J Ethnopharmacol 110:490–497

Kim JY, Kwon O (2011) Culinary plants and their potential impact on metabolic overload. Ann N Y Acad Sci 1229:133–139

Kim KH, Moon E, Choi SU, Pang C, Kim SY, Lee KR (2015) Identification of cytotoxic and anti-inflammatory constituents from the bark of *Toxicodendron vernicifluum* (stokes) FA Barkley. J Ethnopharmacol. doi:10.1016/j.jep.2015.01.024

Kitts DD, Lim KT (2001) Antitumorigenic and cytotoxic properties of an ethanol extract derived from *Rhus verniciflua* stokes (RVS). J Toxicol Environ Health A 64:357–371

Kwak S, Ku SK, Bae JS (2014) Fisetin inhibits high-glucose-induced vascular inflammation in vitro and in vivo. Inflamm Res 63:779–787

Lee J-C, Lee K-Y, Kim J, Na C-S, Jung N-C, Chung G-H, Jang Y-S (2004) Extract from *Rhus verniciflua* stokes is capable of inhibiting the growth of human lymphoma cells. Food Chem Toxicol 42:1383–1388

Lee DS, Jeong GS, Li B, Park H, Kim YC (2010) Anti-inflammatory effects of sulfuretin from *Rhus verniciflua* stokes via the induction of heme oxygenase-1 expression in murine macrophages. Int Immunopharmacol 10:850–858

Lee YR, Hwang JK, Koh HW, Jang KY, Lee JH, Park JW, Park BH (2012) Sulfuretin, a major flavonoid isolated from *Rhus verniciflua*, ameliorates experimental arthritis in mice. Life Sci 90:799–807

Lim KT, Hu C, Kitts DD (2001) Antioxidant activity of a *Rhus verniciflua* stokes ethanol extract. Food Chem Toxicol 39:229–237

Pal HC, Athar M, Elmets CA, Afaq F (2015) Fisetin inhibits UVB-induced cutaneous inflammation and activation of PI3K/AKT/

NFkappaB signaling pathways in SKH-1 hairless mice. Photochem Photobiol 91:225–234

Park MH, Kim IS, Kim S-A, Na C-S, Hong CY, Dong M-S, Yoo H-H (2014) Inhibitory effect of *Rhus verniciflua* stokes extract on human aromatase activity; butin is its major bioactive component. Bioorg Med Chem Lett 24:1730–1733

Shin JS, Park YM, Choi JH, Park HJ, Shin MC, Lee YS, Lee KT (2010) Sulfuretin isolated from heartwood of *Rhus verniciflua* inhibits LPS-induced inducible nitric oxide synthase, cyclooxygenase-2, and pro-inflammatory cytokines expression via the down-regulation of NF-kappaB in RAW 264.7 murine macrophage cells. Int Immunopharmacol 10:943–950

Son Y-O, Lee K-Y, Lee J-C, Jang H-S, Kim J-G, Jeon Y-M, Jang Y-S (2005) Selective antiproliferative and apoptotic effects of flavonoids purified from *Rhus verniciflua* stokes on normal versus transformed hepatic cell lines. Toxicol Lett 155:115–125